THE UNIVERSITY OF ALBERTA

The Purification and Biophysical Characterization of a Porcine Parvovirus

by

© John A. Lynch

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

Virology

Medical Bacteriology

EDMONTON, ALBERTA

SPRING, 1981

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled The Purification and Biophysical Characterization of a Porcine Parvovirus submitted by John A. Lynch in partial fulfilment of the requirements for the degree of Master of Science in Virology.

R. Marusyk
Supervisor
James Brunton
J.W. Carmichael
David C. Secord

Date Dec. 30, 1980

To my love, my life - my wife.

ABSTRACT

The purification and biophysical properties of a porcine parvovirus (PPV) propagated in a cell line were investigated.

PPV replication, as detected by the production of cytopathic effects and viral hemagglutinin, occured only in porcine cells. Electron microscopic examination of PPV-infected swine testicle (ST) cells revealed large numbers of full and empty viral particles as well as amorphous aggregates of soluble viral components in the nucleus.

Viral infectivity could be reliably and objectively titrated by the TCID50 and plaque assay methods. A one step growth curve of PPV in ST cells indicated a latent period of 12-16 h. Maximum infectivity occured at 72 h post-infection, coinciding with the presence of obvious cytopathic effect. 90% of the viral infectivity remained associated with the lysed cellular debris.

A purification procedure involving the gentle lysis of infected cells in the presence of dilute alkaline buffer, clarification of the extract by centrifugation, precipitation of virus in 25 mM Ca^{++} and finally sedimentation to equilibrium in a sucrose-CsCl step gradient was developed for this study. The purification procedure recovered only 20% of the total viral hemagglutinin and 10% of the total viral infectivity.

The virus present in such gradients demonstrated a broad range of particle densities, with the largest quantities of virus, as judged by hemagglutinin titration, most frequently occuring at a buoyant density of 1.40 gm/mL.

Soluble viral components, prepared by sucrose-CsCl centrifugation, were associated with high hemagglutinating activity. Analysis of the soluble component material by immunoelectrophoretic methods indicated the presence of two antigens with differing electrophoretic mobilities but sharing a common antigenic determinant.

SDS-PAGE analysis of PPV purified in this study revealed three polypeptides with molecular weights of 87,000, 69,000 and 66,000.

Analysis of isolated virion nucleic acid by thermal denaturation, electron microscopy and acridine orange staining indicated a single-stranded configuration. The isolated nucleic acid was resistant to RNAse treatment. Electron microscopic and electrophoretic techniques indicated the presence of a wide range of DNA size classes with a maximum size of 1.05 - 1.7 x 10^6 daltons.

Serological studies determined that 76% of market age swine in the Edmonton area had been previously infected with PPV.

Hard work may have bitter roots,
But its fruits are sweet.

Anonymous

ACKNOWLEDGEMENTS

The author wishes to acknowledge the counsel and support of Dr. R.G. Marusyk, whose concerned efforts turned an unfortunate predicament into a rewarding experience. In addition, the guidance and encouragement of Dr. M.S. Shahrabadi during the early part of this study is much appreciated. The author sincerely wishes to thank Linda Tsang and Nicholas Chang for their daily assistance, advice and suggestions, which were provided in an atmosphere of enthusiastic curiosity and friendship. Finally,the author is grateful for the positive criticism provided by Dr. J. Brunton and the the technical assistance of Roger Bradley, Errol Prasad and Richard Sherbourne.

Table of Contents

Chapter Page

I. INTRODUCTION 1

II. MATERIALS AND METHODS 18

A. Virus Isolate 18

B. Cell Culture 18

C. Cell Culture Medium 19

D. Virus Propagation 20

E. Virus Purification 20

F. Virus Assay 23

G. Preparation of Hyperimmune Serum 26

H. Hemagglutination 27

I. Hemagglutination-Inhibition 27

J. Growth Curve of PPV in ST Cells 28

K. Susceptibility of Various Cell Lines to PPV 29

L. Ultraviolet Light Absorption Spectrum of PPV ...30

M. Sucrose Sedimentation of PPV Virions 30

N. Immunoelectrophoresis 31

O. Buoyant Density of Virus 32

P. Preparation of Virion DNA 33

Q. Agarose Gel Electrophoresis of PPV DNA 33

R. SDS-Polyacrylamide Gel Electrophoresis of Virion Proteins 34

S. Thermal Denaturation of Purified Viral DNA 35

T. Electron Microscopy Studies 36

U. Serological Survey 38

V. Buffer Solutions 38

W. Manuscript Production 38

III. RESULTS 39

A. Susceptibility of Various Cell Lines to PPV Infection 39

B. Cytopathological and Morphological Alterations Produced by PPV in Cultured Swine Testicle Cells as Observed by Light and Electron Microscopy 39

C. Purification and Concentration of PPV 46

D. Infectivity Titration 49

E. Growth Curve 54

F. Hemagglutination 59

G. Hemagglutination-Inhibition 60

H. Countercurrent Immunoelectrophoresis 60

I. Rocket Immunoelectrophoresis 63

J. Two-Dimensional Immunoelectrophoresis of PPV Soluble Components 64

K. Ultraviolet Light Absorption Spectrum of Purified PPV 67

L. Sucrose Gradient Sedimentation of Purified PPV .70

M. SDS-Polyacrylamide Gel Electrophoresis of Purified PPV 70

N. Alkaline Sucrose Gradient Sedimentation of PPV DNA 76

O. Thermal Denaturation Curve 76

P. Agarose Gel Electrophoresis of PPV DNA 79

Q. Electron Microscopic Observation of Virion Morphology 80

R. Electron Microscopic Observation of Purified PPV DNA 80

S. Serological Survey 85

IV. DISCUSSION 88
BIBLIOGRAPHY 107

LIST OF TABLES

Table Page

1. Chronology of Discovery of Parvoviruses 2
2. Purification of Porcine Parvovirus by the Modified Tattersall Procedure 22
3. The Production of Cytopathic Effect and Hemagglutinin by Attempted Porcine Parvovirus Infection of Cell Lines from Various Species 40
4. Determination of the Efficiency of PPV Purification by the Modified Tattersall Procedure 52
5. A Serological Survey of Sera from Swine in Central Alberta to Detect Antibodies to PPV 86

LIST OF FIGURES

Figure No. Page

1. Proposed Maturation Scheme for Parvovirus Complete Particles 14
2. Unstained Swine Testicle Monolayer 43
3. Unstained Swine Testicle Monolayer Infected with PPV .43
4. ST Cells Infected with PPV and Stained with Hematoxylin and Eosin 45
5. Electron Photomicrograph Section of ST Cells Infected with PPV 45
6. Electron Photomicrograph Section of ST Cells Infected with PPV 48
7. CsCl-Sucrose Gradient Centrifugation of Calcium Precipitated PPV 51
8. Hemagglutination Profile of Serial Fractions from PPV CsCl-Sucrose Gradient Centrifugation 51
9. Plaques Formed by PPV in ST Cells 56
10. One Step Growth Curve of PPV in ST Cells 58
11. Countercurrent Immunoelectrophoresis of PPV Soluble Components 62
12. Rocket Immunoelectrophoresis of Fractions Collected from CsCl Isopycnic Centrifugation of PPV 66
13. Two-Dimensional Immunoelectrophoresis of PPV Soluble Components 66
14. Ultraviolet Light Absorption Spectrum of PPV 69
15. Sucrose Sedimentation of PPV Virions 69

16. SDS-Polyacrylamide Gel Electrophoresis of PPV
Polypeptides 72

17. Densitometer Tracing of PPV Polypeptides Separated
by SDS-PAGE 75

18. Alkaline Sucrose Sedimentation of PPV DNA 78

19. Thermal Denaturation Curve of PPV DNA 78

20. Electron Photomicrograph of Purified PPV Virions 82

21. Electron Photomicrograph of PPV DNA 84

22. Histogram of PPV DNA Length Distribution 84

ABBREVIATIONS

°C	degrees centigrade
CIE	countercurrent immunoelectrophoresis
cm	centimeter
cm^2	square centimeter
CPE	cytopathic effect
2D	two-dimensional immunoelectrophoresis
d	day
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetate
EM	electron microscopy
g	gravitational force
gm/mL	grams per millilitre
h	hour
HA	hemagglutination
HAd5	human adenovirus serotype 5
HEPES	hydroxyethylpiperazine-ethanesulfonic acid
HI	hemagglutination-inhibition
KRV	Kilham rat virus
M	molar
MDCF	modified direct complement fixation test
min	minute
mL	millilitre
mm	millimeter
mM	millimolar
MOI	multiplicity of infection
MVM	minute virus of mice

nm	nanometer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline (with Ca^{++} and Mg^{++})
PFU	plaque-forming unit
PI	post-infection
PPK	primary fetal pig kidney
SDS	sodium dodecyl sulfate
sec	second
SMEDI	stillbirth, mummification, embryonic death, infertility
SSC	saline sodium citrate
ST	swine testicle
TCID50	50% tissue culture infectious dose
TE	Tris-EDTA
TNE	Tris-NaCl-EDTA
Tris	trishydroxymethylaminomethane
u	micron
ug	microgram
uL	microlitre
v	volts
v/v	volume per volume
w/v	weight per volume

I. INTRODUCTION

The family Parvoviridae was not sufficiently characterized to justify its classification by the International Committee on Nomenclature of Viruses until 1973 (Bachman et al, 1975), although the viral etiology of certain parvoviral diseases has been evident for at least half a century (Verge and Cristoforoni, 1928). The term parvovirus (*Latin*, small) proposed by Lwoff and Tournier in 1966 was chosen over Mayor and Melnick's (1966) more descriptive, but unpalatable, picodnavirus (*Italian*, small; deoxyribonucleic acid) to designate an extremely uniform, unique genus of pathogenic viruses of vertebrates within this family.

During the early 1960's, numerous viruses were tentatively placed in this group (Table 1). The Kilham rat virus was the best studied member during the early years, and the criteria for the classification of parvoviruses are now based on this type species.

Viruses of the family Parvoviridae are small (18-26 nm diameter), non-enveloped, isometric viruses with icosahedral symmetry that replicate in the nucleus of infected cells. Virions contain single-stranded linear deoxyribonucleic acid (DNA) of approximately 1.5-2.2 million daltons (Bachman et al, 1979). The genus Parvovirus is differentiated from the other two genera of the family by its distinctive mode of replication, which unlike the adeno-associated viruses (AAV's) does not require a helper virus and unlike both the AAV's and Densoviruses results in almost exclusive

Table 1

Chonology of Discovery of Parvoviruses

Virus	Reference
Mink Enteritis Virus	Wills (1952)
Rat Virus (C)	Kilham and Oliver (1959)
H-1 Virus (C)	Toolan et al (1960)
H-3 Virus	Dalldorf (1960)
Bovine Parvovirus (C)	Abianti and Warfield (1961)
L-S Virus	Lum and Schreiner (1963)
X14 Virus	Payne et al (1963)
HT Virus	Toolan(1964)
HB Virus	Toolan (1964)
Feline Panleukopenia Virus (C)	Johnson (1965)
Minute Virus of Mice (C)	Crawford (1966)
Porcine Parvovirus (C)	Mayr and Mahnel (1966)
Hemorrhagic Encephalopathy of Rats Virus	El Dadah et al (1967)
Goose Hepatitis Virus (C)	Derzsy (1967)
Minute Virus of Canines	Binn et al (1968)
Kirk Virus	Boggs et al (1970)
KBSH Virus (C)	Hallauer et al (1971)
TVX Virus (C)	Hallauer et al (1971)
Lu III Virus (C)	Hallauer et al (1971)
RT Virus (C)	Hallauer et al (1971)
Norwalk Agent	Dolin et al (1972)
Aleutian Disease Virus of Mink	Porter and Larsen (1974)
Lapine Parvovirus	Matsunaga et al (1977)

Modified from Rose (1974)

(C) classification confirmed by the Study Group on Parvoviridae, Co-ordinating Subcommittee, International Committee on Taxonomy of Viruses (Bachman et al, 1979) encapsidation of only the minus strand of DNA.

The first report of a virus isolate of porcine origin with characteristics of the parvovirus genus was published in 1966 by Mayr and Mahnel following an attempt to isolate the then poorly characterized virus of hog cholera. These researchers had found a non-enveloped, hexagonal virion of 20-22 nm diameter in infected porcine tissue culture. Cartwright et al (1967) reported isolation in 96 of 111 cases of porcine reproductive failure of small serologically identical, hemagglutinating viruses, which on further study (Cartwright et al, 1969; Cartwright et al, 1971) showed similar properties and serological reactivities to the earlier German isolate. After a series of similar reports from various sources in Europe (Huygelen and Peetermans, 1967; Darbyshire and Roberts, 1968; Mayr et al, 1968; Johnson, 1969; Johnson and Collings, 1969; Bachman, 1970; Johnson and Collings, 1971), isolations were recorded from Asia (Morimoto et al, 1972a), North America (Mengeling, 1972), and Africa (Pini, 1975). It was not until 1977 that porcine parvovirus (PPV) was reported in Canada (Dulac et al, 1977).

In 1971 Hallauer et al published the results of a ten year investigation into the significance of parvoviruses as contaminants of various cell lines of human and non-human origin. They detected contamination in 27 of 41 cell lines examined by a parvovirus serologically related to porcine parvovirus. This group postulated that contamination arose from use of porcine pancreatic trypsin, that is filtered but not autoclaved prior to use. In support of this hypothesis, Croghan et al (1973) reported the successful recovery of PPV from a batch of commercially prepared trypsin.

Porcine parvovirus has been isolated from swine suffering from a wide range of clinical disease states including rhinitis (Mengeling, 1972), lower respiratory infections (Darbyshire and Roberts, 1968), myoclonia, harelip, splay-leg, atrophy of intestinal mucosa (Johnson and Collings, 1971), mummification (Mengeling,1975), infertility, abortions, stillbirth, neonatal deaths (Cartwright et al, 1971) as well as apparently healthy piglets (Dulac et al, 1977). However, it is only with viral-induced reproductive failure, the so-called SMEDI syndrome (stillbirth, mummification, embryonic death, infertility) that PPV is routinely and reproducibly associated at present.

The role of PPV in the SMEDI syndrome has been verified both by field study of naturally occurring infections and studies of artificially induced infections. Apart from the multiple reports of PPV isolation cited previously from mummified or stillborn fetuses, the most incriminating reports of reproductive failure following natural transmission of PPV have been contibuted by Donaldson-Wood et al (1977) and Rodeffer et al (1975). Donaldson-Wood et al found that a herd of PPV seronegative swine being regularly monitored for evidence of PPV infection suddenly had four animals seroconvert. Within two months all mature swine on the premises had become seropositive. Two weeks later an outbreak of fetal mummification, stillbirth, low litter size and neonatal deaths began and continued for five weeks. Six of fifteen pregnant sows produced mummified fetuses, with virus isolation being successful in several cases. Rodeffer et al (1975) noted similar findings when seronegative sows were exposed to seropositive boars. In addition they noted that reproductive performance was again adequate with subsequent litters.

Numerous reports have been published concerning attempts to experimentally produce PPV-induced reproductive failure (Johnson and Collings,1969; Johnson and Collings, 1971; Cartwright et al, 1971; Redman et al, 1974; Bachman et al, 1975; Cutlip and Mengeling, 1975a; Cutlip and Mengeling, 1975b; Mengeling and Cutlip, 1975; Joo et al. 1976c; Hogg et al, 1977; Mengeling, 1979; Mengeling et al, 1979). The consensus from these reports indicates that infection during the early part of the second third of gestation is most likely to result in fetal death and mummification. Infection early in the third part of gestation may produce death with antibody formation. In the middle of the third part of gestation the fetus' immune system may be sufficiently developed to prevent death but virus and antibody can often be isolated at birth. In the last part of the final third of gestation fetuses generally do not show evidence of infection and anti-PPV antibodies are not detected. In the first third of gestation infection can lead to fetal death with resorption of fetal tissues but retension of corpora lutea, resulting in low litter size or pseudopregnancy. Alternatively, fetuses may show no evidence of infection but continue on to term in a state of tolerance for long periods of time and excrete virus for several months. The ability of virus to spread from one infected fetus to a non-infected fetus has been observed to be quite variable. In some cases virus spreads readily and induces death, in others antibodies and/or virus can be isolated and in still others no spread whatsoever has been detected. The presence of a non-antibody anti-parvovirus glycoprotein substance has been isolated from human placenta (Usategui-Gomez, 1965) but such substances have not been reported in porcine placentas.

Comparison of all strains of PPV examined to date has proven these strains to be serologically identical on the basis of hemagglutination-inhibition (HI), serum neutralization (SN) and modified direct complement fixation (MDCF) tests (Cartwright et al, 1969; Johnson, 1973; Ruckerbauer et al, 1978). Comparison of parvoviruses from various species by serological methods has indicated that distinct subgroups exist (Hallauer et al, 1971; Joo et al, 1976d). The only virus to show any cross-reaction with PPV by HI and SN tests is the KBSH virus. Although these two viruses exhibit some apparent relationship, the distinct differences in their range of host cell susceptibilities indicate a significant difference (Cartwright et al, 1969;

Hallauer et al, 1971). Serological studies using highly purified viral antigens and antiserum produced in response to such antigens have not been described for PPV and the results of the above studies might be questionable.

PPV has been most frequently and successfully propagated in primary fetal pig kidney (PPK) cell cultures (Mayr et al, 1968; Cartwright et al, 1969; Mengeling, 1972; Johnson, 1973). Although some researchers have successfully grown PPV in cell lines (Mayr et al, 1968; Bachman, 1972), this method has only been used for determination of relative hemagglutinin and infectivity production with respect to time. All reported attempts to propagate PPV in cells of non-porcine origin have been unsuccessful (Cartwright et al, 1969; Mayr et al, 1968). The trophic requirement of parvoviruses in general for actively dividing cells (Rose, 1974) has been similarly demonstrated for PPV (Cartwright et al, 1969; Bachman 1972). Attempts to isolate PPV on confluent monolayers of PPK cells has proven unsuccessful when oral dosing of the same material to gnotobiotic pigs has resulted in consistent seroconversion (Redman et al, 1974). The production of cytopathic effect (CPE) as a result of successful infection in tissue culture is reported to occur only under optimal conditions with high virus challenge (Joo and Johnson, 1976).

A chronological study of PPV growth in a pig kidney (PK15) cell line (Mayr et al, 1968) demonstrated viral hemagglutinin as early as 6 hours (h) post-infection (PI) with a peak to a plateau at 72 h. Infectivity was first detected at 15 h and did not peak until 96 h. A later study of PPV growth in the swine kidney (SK) cell line (Bachman, 1972) showed similar onsets for hemagglutination (HA) activity and infectivity but both peaked quite rapidly at 24 h after infection. This work also demonstrated that viral replication was suppressed at 40 degrees centigrade (°C) and that replication was highly associated with the mitotic index of the monolayer. A study of PPV replication in peripheral blood lymphocytes, monocytes and peritoneal macrophages revealed a growth curve similar to that demonstrated by Mayr et al (1968) in mitogen stimulated lymphocytes but not in unstimulated lymphocytes or monocytes or macrophages, as assessed by HA production and fluorescent antibody staining of cells (Paul et al, 1979).

An attempt to purify PPV has only been reported in two publications (Mayr et al, 1968; Morimoto et al, 1972b). Mayr et al used repeated freeze-thawing of infected cultures to release cell-bound virus. After centrifugation to remove cellular debris, the supernatant was concentrated tenfold by vacuum evaporation and the virus was then pelleted by ultracentrifugation. The pellet was resuspended and sedimented through a CsCl step gradient for 14-16 h. No assessment of the degree of purity was attempted other than electron microscopic (EM) observation. Morimoto et al similarly relied on isopycnic CsCl centrifugation of infected cell lysates to achieve purity and did not report any assessment of the degree of purity achieved.

A wide range of techniques have been reported for purification of various parvoviruses, with that described by Tattersall et al (1976) being most frequently employed in the more recent literature. Freeze-thawing, detergent treatment, sonication and mechanical homogenization have all been described as initial steps to disrupt infected cells (Bloom et al, 1980; Rhode, 1973; Richards et al, 1977; Tattersall et al, 1976). Disruption of virion bonds to cellular material as a subsequent step has been accomplished by the use of dilute alkaline buffer, freon extraction and enzyme treatment (Siegl et al, 1971; Tattersall, 1972; Bourgignon et al, 1976). Separation of dissociated virions and cellular remnants has been reported by sucrose sedimentation, CsCl isopycnic centrifugation, or sedimentation to equilibrium in a combined sucrose and CsCl step gradient (Richards et al, 1977; Salzman and Koczot, 1978; Patton et al, 1979).

The buoyant density for PPV at equilibrium in CsCl gradients is reported to be 1.38 grams per millilitre (gm/mL) (Mayr et al, 1968; Morimoto et al, 1972b). Virions have been observed with densities as high as 1.44 gm/mL (Mayr et al, 1968), and empty appearing particles with a density of 1.30 gm/mL have also been observed (Morimoto et al, 1972b). No attempt has been made to classify these various density classes of particles by polypeptide or nucleic acid content. The value of 1.38 gm/mL is somewhat below the more commonly reported range of 1.40-1.42 gm/mL for parvoviruses in general (Bachman et al, 1979). Studies on other parvoviruses have revealed three basic particle density classes: empty particles, banding at 1.30-1.32 gm/ml; light full particles, banding at 1.40-1.42 gm/ml; and heavy full particles, banding at 1.42-1.44 gm/mL (Tattersall, 1978a). In addition, a very heterogeneous population of particles with less than a complete genome banding between 1.32 and 1.38 gm/mL has also been demonstrated (Bourgignon et al, 1976; Faust and Ward, 1979).

In common with findings for other parvoviruses, PPV has been repeatedly demonstrated to be resistant to inactivation by a wide range of pH, temperature, lipid solvents and trypsin. Studies by Cartwright et al (1969) showed that treatment with ethyl ether for 18 h at 4°C or pH 3.0 for 3 h at 37°C resulted in no significant loss of HA titre or infectivity compared to a control. Also, this same group demonstrated that virus held at -20°C or -70°C for six months had little or no decrease in HA titre and that virus held at 70°C for 2 h, 56°C for 48 h and 37°C for 7 days (d) was still infective. However, infectivity was lost after 5 minutes (min) at 80°C. Work by Morimoto et al (1972b) confirmed the above and in addition indicated that PPV was resistant to treatment with chloroform, sodium deoxycholate and trypsin. Mayr et al (1968) also reported similar findings but noted that incubation at pH 2 for 90 min at 37°C completely destroyed infectivity. Mengeling (1972) as well, in similar studies, confirmed the extreme stability of this virus.

Hemagglutination is a consistent property of parvoviruses, with the range of species whose erythrocytes are susceptible to agglutination being characteristic of the species of parvovirus (Bachman et al, 1979). The spectrum of erythrocytes that can be agglutinated by PPV has been investigated in numerous studies (Huygelen and Peetermans, 1967; Mayr et al, 1968; Cartwright et al, 1969; Morimoto et al, 1972b; Mengeling, 1972; Johnson, 1973; Joo et al, 1976b). No contradictions in this spectrum have been reported for any of the various strains studied, although minor differences in the relative titres produced with different erythrocytes have been noted (Joo and Johnson, 1976). PPV has been shown to agglutinate erythrocytes from man (type O), guinea pig, monkey, rat, mouse, chicken, and cat but not horse, pig, sheep, cattle, goat, or duck. The previously cited studies have all concluded that optimal results are obtained with guinea pig erythrocytes at physiological pH, 4°C for 2 to 18 h. Hemagglutination by subviral components has not been reported, as it has for certain other hemagglutinating viruses (Norrby, 1966).

Purified parvoviruses have been shown to produce a consistent and characteristic polypeptide pattern when virion protein is disrupted and analysed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (Tattersal, 1978a). However, the polypeptide structure of

PPV has not yet been described. Polypeptide A (Tattersal et al, 1976), the largest of the three regularly reported polypeptides, has been variously reported as having a molecular weight of 72-92,000 for different parvovirus species and to consistently comprise 15-18% of all virion classes. Polypeptide B, the predominant polypeptide in empty and heavy full particles has been shown to have a molecular weight ranging from 64-77,000. Polypeptide C is the predominant polypeptide in light full particles. This polypeptide is believed to be derived from a post-translational enzymatic cleavage of the B polypeptide of full heavy particles (Figure 1) (Clinton and Hayashi, 1976). The molecular weight of polypeptide C is reported to be 61-69,000. In some parvoviral systems a fourth polypeptide is described with molecular weight 40-56,000. Whether this D polypeptide is a further cleavage product or a cellular contaminant has not adequately been assessed. However it has been determined that its presence is not required for infectivity (Tattersall et al, 1976).

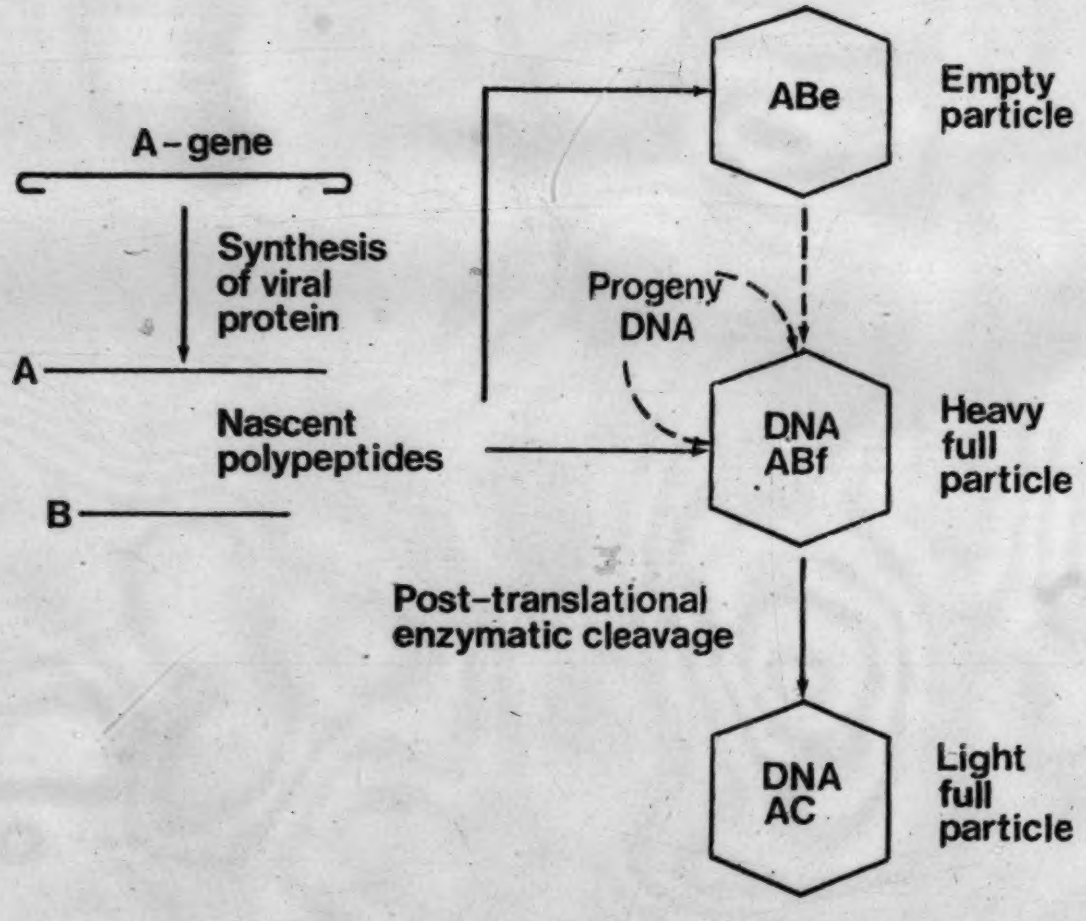
Although it is generally agreed that the structural protein of parvoviruses has no DNA polymerase activity, the matter is still open for debate and it is thought that it may play some important role in nucleotide chain elongation or DNA packaging (Berns and Hauswirth, 1978). Salzman and Koczot (1978) demonstrated that when purified Kilham rat virus (KRV) was disrupted at pH 12.2 for 5 min and then sedimented through an alkaline sucrose gradient pH 10, DNA

Figure 1

Proposed Maturation Scheme for Parvovirus Complete Virions

A, B and C denote the three viral polypeptides. Suffixes denote the postulated B-C cleavage-resistant (e) or cleavage-sensitive (f) conformations of the particle. Empty, heavy full and light full describe virion classes.

(Adapted from Tattersall, 1978a).


A-gene
Synthesis of viral protein
A
Nascent polypeptides
B
ABe
Empty particle
Progeny DNA
DNA ABf
Heavy full particle
Post-translational enzymatic cleavage
DNA AC
Light full particle

polymerase activity was associated with a radioactively labelled fragment sedimenting at 8 to 10 S. If the virus was sedimented through a gradient at pH 12.2 the activity was lost. Rhode (1973) was unable to demonstrate polymerase activity associated with the parvovirus H-1 or KRV. Bates et al (1978) have shown that viral DNA replication closely parallels increases in cellular alpha polymerase levels. Bates et al have also shown that polymerase is only associated with virus purified by the less extensive procedure of Salzman and Jori (1970) and not with virus purified by the more complex procedure of Tattersall et al (1976). Virions purified by the Tattersall procedure are completely infectious and therefore Bates et al have concluded that the polymerase activity demonstrated is, in fact, due to a contaminating cellular polymerase.

The nature of the nucleic acid of PPV has been insufficiently studied (at the present time) to conclusively justify its classification as a parvovirus as established by Bachman et al (1979). Identification of the nucleic acid type by the use of a halogenated thymidine analog or actinomycin D acting as known inhibitors of DNA replication has in most cases indicated a DNA genome (Mayr et al,1968; Cartwright et al, 1969; Mengeling, 1972; Morimoto et al, 1972b) but not unambiguously (Huygelen and Peetermans, 1967). The results of feulgen staining of PPV infected cells has also indicated a DNA genome (Cartwright et al, 1969).

The DNA of most currently accepted parvoviruses has been shown to be a single-stranded linear molecule with non-identical hairpin termini by a variety of physico-chemical methods, enzymatic treatments and electron microscopy (Berns and Hauswirth, 1978). The DNA of KBSH, the only virus demonstrating a close serological relationship to PPV, has been studied in some detail by Siegl (1972). Although the exact nature of this relationship is still poorly defined, this data has apparently been accepted for PPV as well (Bachman et al, 1979). From the sedimentation co-efficient in neutral sucrose gradients and the minor variation in S values for native and denatured DNA, Siegl has deduced that KBSH virus has a single-stranded DNA genome of approximately 1.4 million daltons in size. This same author noted that incomplete particles banding at a lower density were routinely evident. These particles contained a heterogeneous distribution of DNA lengths, most frequently comprising 20% of the complete genome. Similar results have been documented for several other parvoviruses (Shahrabadi et al, 1977; Rhode, 1978; Faust and Ward, 1979).

Only two limited studies have been attempted to investigate the value of vaccination in preventing PPV induced reproductive failure. Studies of serological response to an inactivated vaccine produced by Joo and Johnson (1977), indicated that antibody titres were sufficient to assure protection for a period of six months. Mengeling et al (1979) in a more detailed study of a low number of a animals showed that a presumably attenuated strain of PPV inactivated by acetylethyleneimine protected vaccinates from challenge by oro-nasal administration of an assumed virulent strain. Non-vaccinated controls all produced dead fetuses with successful virus isolation from each litter. No virus was isolated from piglets born to vaccinated sows and no antibody detected.

In light of the foregoing, the present project was undertaken with two goals. The first was to satisfy some of the outstanding requirements of nucleic acid and protein characterization to unquestionably classify PPV as a parvovirus. The second was to investigate the feasibility of growth and purification of PPV in a defined cell line system, as opposed to less convenient primary fetal cells. Such a system hopefully would prove suitable for commercial vaccine production, reliable viral isolation and quantitation and the production of high quality diagnostic reagents.

II. MATERIALS AND METHODS

A. Virus Isolate

The virus isolate used in this study was the ADRI-1 strain of PPV isolated in Ottawa, Canada, from a primary porcine thyroid cell culture (Ruckerbauer et al, 1978). The virus was received as a gift from Dr. H. J. Cho (Animal Disease Research Institute, Lethbridge, Alberta, Canada). This strain has been found to be serologically indistinguishable from two other well studied isolates (59E/63, Cartwright et al, 1969; G 10/1, Mayr et al, 1968) by the modified direct complement-fixation, Hemagglutination-inhibition and fluorescent antibody tests.

Upon receipt, the virus was passaged in primary porcine fetal kidney cells prior to further study and the medium from infected cultures was stored at -20°C until required.

B. Cell Culture

A swine testicle (ST) cell line was used almost exclusively during this study to propagate PPV. This cell line was chosen for its ability to support the growth of the virus with a distinct cytopathic effect. Information concerning the passage history and origin of the cell line were unavailable from the donor, Dr. J. F. Pantekoek (Alberta Provincial Veterinary Laboratory, Edmonton, Alberta, Canada) and could not be traced. With reference to cellular morphology, growth characteristics and an aneuploid karyotype, it was suspected that the ST cells were derived from an epithelial neoplasm (Figure 2).

Upon receipt the ST cells were found free of mycoplasma by culture methods. At various intervals during this study, checks were made for endogenous or contaminating parvoviruses by the method of Hallauer and Kronauer (1965) and were consistently found negative.

Primary cells were prepared by trypsinization of freshly excised fetal porcine kidneys using standard methodology (Rovozzo and Burke, 1973).

C. Cell Culture Medium

Medium for both growth and infection was prepared from a powdered minimum essential medium base (MEM-Eagle, modified Auto-Pow, Flow Laboratories, Inc., Inglewood, Ca.) which was dissolved in distilled water. The medium was autoclaved for 15 min at 15 psi, 120°C and stored at 4°C until required.

Calf serum was added to growth medium to a level of 5% [volume per volume (v/v)] prior to use. Filter sterilized solutions of glutamine, bicarbonate, penicillin and streptomycin were added to final concentrations of 2 millimolar (mM), 0.1% [weight per volume (w/v)], 100 international units/mL and 200 micrograms (ug) per mL, respectively.

For the preparation of medium to be used during infection, fetal calf serum at the same concentration was substituted for calf serum.

D. Virus Propagation

For routine virus production, ten or twenty 150 square centimeter (cm^2) glass culture (Blake) bottles were seeded with approximately 7 x 10^6 ST cells per bottle and incubated at 37°C When monolayers were 50% confluent, with minimal cell to cell contact and appeared to be entering the log phase of growth, 36 to 48 h later, the growth medium was removed. The cell surface was washed twice with sterile phosphate buffered saline with Ca^{++} and Mg^{++} followed by the addition of 10 mL of virus inoculum diluted in PBS to a multiplicity of infection (MOI) of 10^{-3} plaque-forming units (PFU) per cell. The bottles were then incubated at 37°C for 1 h, with gentle swirling each 15 min. After the adsorption period, 100 mL of infection medium was added to each bottle and incubation was continued at 37°C until CPE was 75% complete, generally in 3-5 d.

E. Virus Purification

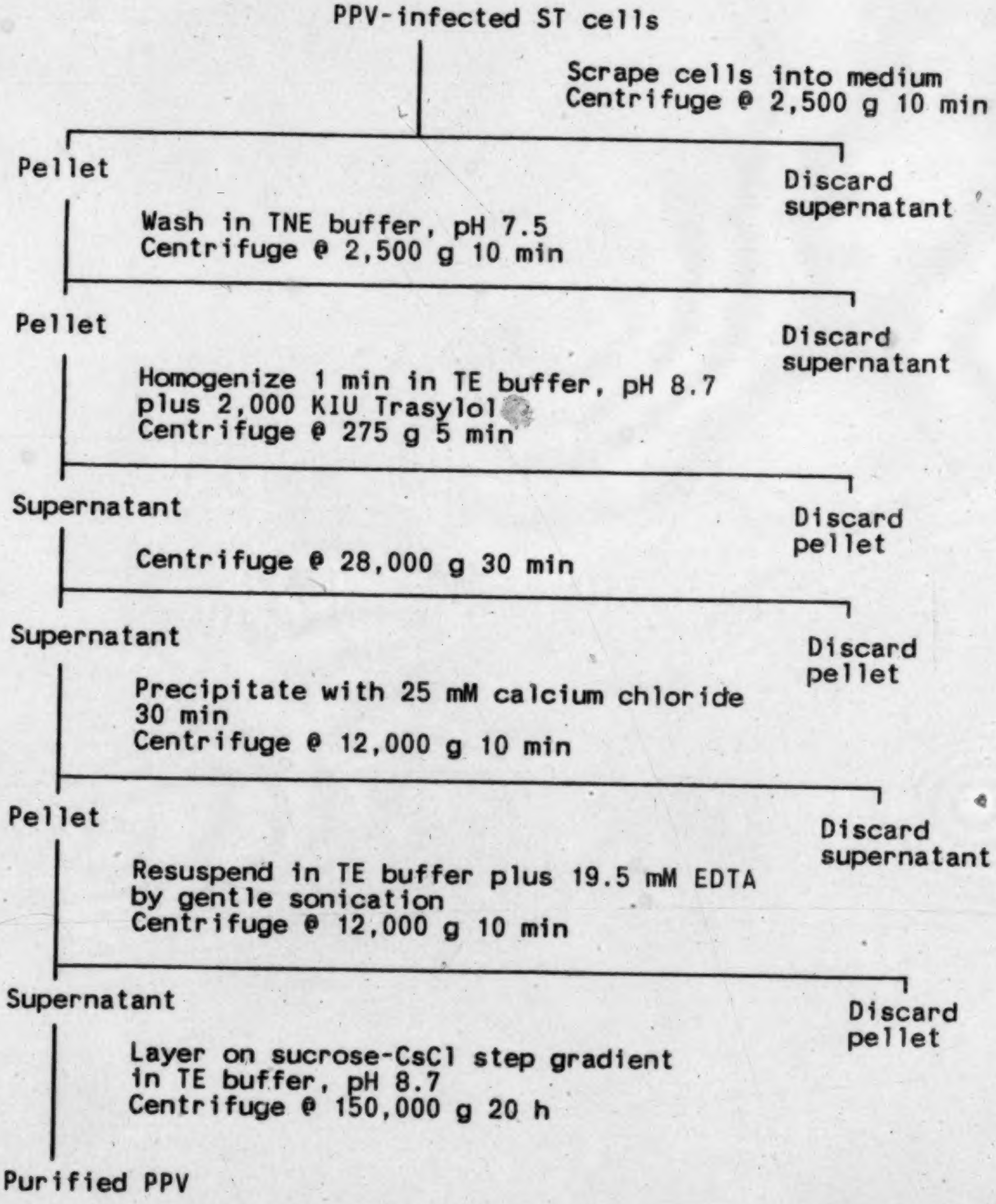
Infected cells were scraped into the medium with a rubber policeman and centrifuged at 2,500 gravitational forces (g) for 10 min. The cell pellet was washed once in neutral TNE buffer [50 mM trishydroxymethylamino-methane-hydrochloride (Tris); 150 mM NaCl; 0.5 mM ethylenediamine tetra-acetate (EDTA)] and similarly repelleted. Purification proceeded by one of two methods from this point.

a) The cell pellet was resuspended in 50 mM glycine buffer, pH 9.0, and sonicated at low frequency for four 15 second (sec) bursts at 0-4°C. The cell suspension was incubated at 4°C for 1 h and then twice extracted with an equal volume of Freon 113 (trichlorotrifluoroethane; Terochem Laboratories Ltd, Edmonton, Canada) with centifugation at 3,000 g to separate the aqueous and non-aqueous phases. Particulate cellular debris was removed from the upper aqueous phase by centrifugation at 12,000 g for 10 min. The supernatant was layered onto a step gradient consisting of 2 mL of 1.0 molar (M) sucrose above 5.0 mL of a CsCl solution, buoyant density 1.40 gm/mL (both solutions being prepared in the above glycine buffer). The virus suspension was sedimented to equilibrium in a SW40 rotor at 150,000 g for 20 h in a Beckman L5-75 ultracentrifuge (Beckman Insts., Palo Alto, Ca.).

b) The cell pellet was suspended in TE buffer (50 mM Tris; 0.5 mM EDTA; pH 8.7) and 2,000 kilo-international units Trasylol (FBA Pharmaceuticals, Distributor Boehringer Ingelheim, Dorval, Que.) was added (Table 2). The cell suspension was then homogenized in a Sorvall Omni-mixer (Dupont Insts., Wilmington, Delaware) for one minute on ice. The suspension was centrifuged immediately at 275 g for 5 min, and the supernatant at 28,000 g for 30 min. The resulting supernatant was adjusted to 25 mM calcium chloride and incubated at 4°C for 30 min. The precipitate was

Table 2

Purification of Porcine Parvovirus by the Modified Tattersall Procedure



PPV-infected ST cells

Scrape cells into medium
Centrifuge @ 2,500 g 10 min

Pellet

Discard supernatant

Wash in TNE buffer, pH 7.5
Centrifuge @ 2,500 g 10 min

Pellet

Discard supernatant

Homogenize 1 min in TE buffer, pH 8.7
plus 2,000 KIU Trasylol
Centrifuge @ 275 g 5 min

Supernatant

Discard pellet

Centrifuge @ 28,000 g 30 min

Supernatant

Discard pellet

Precipitate with 25 mM calcium chloride
30 min
Centrifuge @ 12,000 g 10 min

Pellet

Discard supernatant

Resuspend in TE buffer plus 19.5 mM EDTA
by gentle sonication
Centrifuge @ 12,000 g 10 min

Supernatant

Discard pellet

Layer on sucrose-CsCl step gradient
in TE buffer, pH 8.7
Centrifuge @ 150,000 g 20 h

Purified PPV

pelleted at 12,000 g for 10 min and the supernatant discarded. TE buffer plus 19.5 mM EDTA was added and the pellet resuspended by vortex agitation and gentle sonication. Insoluble material was removed by further centrifugation at 12,000 g for 10 min, and the viral suspension layered onto a step gradient as above (sucrose and CsCl prepared in TE buffer) and similarly centrifuged.

Following centrifugation, fractions were collected dropwise by bottom puncture for further assessment or visible bands were removed by pipette and studied directly.

F. Virus Assay

Virus infectivity was measured by several methods:

a) 50% Tissue Culture Infectious Dose Assay (TCID50). Meticulously cleaned glass culture tubes were seeded with 5 x 10^5 ST cells in growth medium and incubated overnight at 37°C. The following day the culture tubes were examined for the presence of sufficient cell adherence and minimal cell to cell contact. Suitable tubes were then infected in quadruplicate with serial tenfold dilutions of viral inoculum prepared in PBS as described previously. During infection and PI incubation, tubes were placed in a roller apparatus (Labline Insts. Inc., Melrose Park, Ill.) Cell cultures were monitored for CPE for 5 d PI, and the log TCID50 calculated by a modification (Marusyk, personal communication) of the technique of Reed and Muench (1938).

b) Plaque Assay. For plaque assays, 3.5 x 10^5 ST cells were seeded into 60 mm plastic Petri dishes and incubated overnight at 37°C in a 5% carbon dioxide atmosphere in growth medium supplemented with 10 mM hydroxyethylpiperazine-ethansulfonic acid (HEPES). The following morning all dishes were examined for even cell distribution with minimal cell to cell contact. Growth medium was removed and dishes were washed gently with PBS once. Serial tenfold dilutions of virus were prepared in PBS, and duplicate plates inoculated with 0.2 mL each. Plates were incubated for 1 h at 37°C with gentle rotation at 15 min intervals. Following adsorption, the inoculum was removed, and dishes were overlaid with 5 mL of agar medium consisting of: 1 Part: 1.5% (w/v) Noble agar (Difco) in distilled water at 43°C added to 1 Part: 2 x infection medium at 37°C (without phenol red) supplemented with 20 mM HEPES. The medium was allowed to solidify at room temperature for 1 h and the plates were incubated without inversion at 37°C in a 5% carbon dioxide atmosphere. The following day 2 mL of infection medium without phenol red were added to each dish. Dishes were either stained 4 d PI by the addition of 3 mL of agar medium containing 1:10000 neutral red and plaques counted the following day, or were fixed 5 d PI with 10% formaldehyde and stained 2 h later with crystal violet.

c) Inclusion Body Assay. Glass Leighton tubes with acid-cleaned glass coverslips were seeded with 5 x 10^5 ST cells each. The following day tubes were examined and infected in duplicate as described above. At 48 h PI, coverslip monolayers were washed with PBS, fixed overnight with Zenker's solution and stained with hematoxylin and eosin as described by Hsiung (1973). Stained coverslips were mounted on slides and examined by light microscopy. Typical inclusions were counted and the titre determined from the highest dilution showing distinct inclusions.

d) Fluorescent Focus Assay. The procedure described by Thiel and Smith (1967) was modified slightly for use in this study. Petri dishes were seeded and incubated as described for the plaque assays. Duplicate petri dishes were infected at 50% confluence with serial tenfold dilutions of the viral suspension as in the plaque assay. Following infection, 5 mL of infection medium supplemented with 10 mM HEPES was added to each dish after removal of the inoculum. The cultures were incubated in a 5% carbon dioxide atmosphere at 37°C for 48 h. At this time the medium was removed and each plate was washed four times with 0.9% (w/v) NaCl. The monolayers were then fixed by adding 5 mL of 95 volumes methanol:5 volumes 0.9% NaCl. for 4 min. The fixative was removed and the plates were washed with 100 mM phosphate buffer, pH 7.1, for 4 min. The fixed monolayers were then covered with 0.4 mL of PPV antiserum prepared in rabbits as described below. Plates were allowed to stand at room temperature for 30 min with gentle swirling each 10 min. The antiserum was then removed and the plates were washed six times with phosphate buffer. 0.4 mL of a 1:5 dilution of fluorescein isothiocyanate conjugated anti-rabbit immunoglobulin (BBL, Cockeysville,MD) was then added to each plate. After a similar incubation, each plate was washed three times with phosphate buffer. Plates were then examined with a Zeiss Jena microscope with a dark field condenser under ultraviolet illumination. The number of fluorescent cells were counted in the highest dilution producing fluorescence and the titre calculated from the appropriate dilution factor.

G. Preparation of Hyperimmune Serum

Purified PPV was obtained by the Ca^{++} precipitation and sucrose-CsCl centrifugation procedure described above. Virus protein was quantitated by a commercially available kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, Ca.). Virus was disrupted by extended sonication, repeated freeze-thawing in liquid nitrogen and dialysis against distilled water. Disruption was assessed by pre- and post-treatment electron microscopy. Rabbits were injected intramuscularly with approximately 160 ug of whole or disrupted virus that had been dialyzed overnight against PBS and mixed with an equal volume of Freund's incomplete adjuvant. Intravenous boosters of a similar quantity of virus without adjuvant were given four and eight weeks later. Rabbits were test bled one week later and antibody production assessed by countercurrent immunoelectrophoresis (CIE) against homologous antigen. One week after the test bleed, the rabbits were terminally exsanguinated. Sera were partially purified by amonium sulfate precipitation (Axelsen et al, 1973), resuspended in distilled water and stored at -20°C until use.

H. Hemagglutination

Serial two-fold dilutions of viral samples were prepared in PBS for either the micro-(0.05 mL volume) or macro-titration (0.2 mL volume) method in microtitre plates (Cooke Engineering Co., Alexandria, Va.) or test-tubes, respectively. An equal volume of a 1% (v/v) suspension of guinea pig or African green monkey erythrocytes were added to each well or tube and the tests were incubated at 4°C for 2 h. The hemagglutination (HA) titre was recorded as the highest dilution of test sample to cause complete agglutination of the erythrocytes.

I. Hemagglutination-Inhibition

Hemagglutination-inhibition (HI) tests were performed as described by Joo et al (1976b). Non-specific inhibitors of hemaggutination were removed by heat inactivation of test sera at 56°C for 30 min followed by absorption with 25% (w/v) kaolin in PBS. Endogenous hemagglutinins were removed by adsorption with the erythrocytes used in the test for 1 h at room temperature. Treated sera were then serially diluted and mixed with an equal volume of hemagglutinin containing 4 HA units. Following incubation at room temperature for 1 h, an equal volume of 1% guinea pig, or occasionally African green monkey, erythrocytes was added. Tests were then incubated at 4°C for 18 h. The hemagglutination-inhibition titre was recorded as the highest dilution of serum to completely inhibit hemagglutination.

J. Growth Curve of PPV in ST Cells

Forty 25 cm^2 plastic culture flasks were sparsely seeded with ST cells (5 x 10^5 cells/flask) and allowed to settle overnight to produce minimal cell to cell contact. The cells were then parasynchronized by incubation at 4°C for 3 h followed by rapid warming to 37°C before infection.

The cultures were washed once with PBS and infected with 1 mL of a 1:1000 dilution of seed virus (MOI 10^{-3} PFU/cell) prepared in PBS and allowed to adsorb at 37°C for 1 h. Unadsorbed virus was removed by washing each flask three times with PBS. Five mL of infection medium was then added to each flask and incubation was continued at 37°C

At 6 h intervals from 6 to 48 h PI and at 72 and 96 h PI, flasks were examined for CPE and four flasks were selected randomly for determination of intracellular and extracellular infectivity and hemagglutinin. To assess intracellular virus, monolayers were washed once in TNE buffer, cells were scraped from the flask, pooled with cellular debris pelleted from the supernatant medium and suspended in 1 mL of 50 mM glycine buffer, pH 9.0. The cell suspension was frozen and thawed three times and then incubated at 4°C for 1 h to extract virus. All samples were stored at -20°C prior to analysis for HA titres and infectivity (by TCID50 assay) as previously described.

K. Susceptibility of Various Cell Lines to PPV

Attempts were made to infect the following cell lines with PPV:

1) BHK-21 (baby hamster kidney; a gift from Dr. J. Colter, Biochemistry, University of Alberta, Edmonton, Alberta).

2) BSC-1 (African green monkey kidney; ATCC 46403).

3) HeLa (human cervical carcinoma; a gift from Dr. O. Morgante, Bacteriology, University of Alberta, Edmonton, Alberta).

4) HEp-2 (human epidermoid laryngeal carcinoma; Flow Laboratories, Mississauga, Ontario).

5) KB (human nasopharyngeal carcinoma; a gift from Dr. S. Mak, Biology, McMaster University, Hamilton, Ontario).

6) KB pre-infected with human adenovirus serotype 2.

7) LLC RK-1 (rabbit kidney; ATCC 46369).

8) MRC-5 (human lung fibroblasts; ATCC 6388).

9) ST (swine testicle; a gift from Dr. J. Pantekoek, Microbiology, Provincial Veterinary Laboratory, Edmonton, Alberta).

10) Vero (African green monkey monkey; Flow Laboratories Mississauga, Ontario).

Duplicate cultures were seeded in 75 cm^2 plastic culture flasks. When approximately 50% confluence was evident, one flask was infected with 2 mL of a crude PPV preparation diluted in PBS to an approximate MOI of 2 x 10^{-3} PFU/cell. The other culture was left as an uninfected control. The cells were observed daily for CPE. When CPE was maximum or when 5 d had elapsed, the cells were scraped into the medium, pelleted by low speed centrifugation, and washed once in neutral TNE buffer. After centrifugation, the buffer was poured off and the cells were resuspended in 2 mL of 50 mM glycine buffer, pH 9.0, by vortex agitation. The cell suspension was incubated at 4°C for 1 h and then centrifuged once more. The supernatant fluid was assayed for viral hemagglutinin as described previously.

L. Ultraviolet Light Absorption Spectrum of PPV

A suspension of porcine parvovirus purified by Ca^{++} precipitation and sucrose-CsCl centrifugation was tested for ultraviolet light absorption between 235 and 310 nm wavelength. Measurements were performed with a Gilford 250 spectrophotometer (Gilford Inst. Lab., Oberlin, Ohio).

M. Sucrose Sedimentation of PPV Virions

Purified PPV from a CsCl gradient was dialyzed overnight against TNE buffer, pH 7.5. The virus suspension was layered on a 5-20% linear sucrose gradient and centrifuged in a SW40 rotor for 90 min at 150,000 g in a Beckman L5-75 ultracentrifuge. Fractions were collected dropwise by bottom puncture and assayed for hemagglutination titres.

N. Immunoelectrophoresis

a) Countercurrent Immunoelectrophoresis. Glass plates 8 cm x 10 cm were carefully layered with 20 to 25 mL of 1% (w/v) agarose prepared in Tris-glycine buffer (50 mM Tris, 380 mM glycine, pH 8.3). Two rows of wells 4 millimeters (mm) in diameter and spaced 8 mm apart were cut into the agar. The well on the anodal side was filled with 35 uL of test serum and the well on the cathodal side with an equal volume of viral antigen extract. This antigen consisted of the glycine-freon extract described above (see Purification), and was diluted as required to have an HA titre of 4096. Electrophoresis was carried out in the same buffer contained in the agar at 150 v for 1 h in a Bio-Rad model 1415 electrophoresis cell. Precipitation lines were evident immediately following electrophoresis. For permanent records, gels were pressed under wet filter paper, several layers of paper towel, a flat cover and moderate weight. They were then dried to a thin film with a hot air blower, rinsed free of any precipitated salts or spilled reagents in buffer and finally stained with 0.2% Coomassie Brilliant Blue R in methanol:acetic acid:water (5:1:4) and destained in the same solution minus the stain.

b) Rocket Immunoelectrophoresis. Agar plates were prepared as above except that the agar was cooled to approximately 45°C and 50 microlitres (uL) of antiserum against disrupted PPV was added to 25 mL agar prior to pouring the plates. Rows of sample wells were cut into the agar as above and filled with 25 uL samples. Electrophoresis was performed at 250 v for 2-6 h. Following electrophoresis plates were pressed, dried and stained as described above.

c) Two-dimensional Immunoelectrophoresis. Overlapping triplicate 4 mm wells were cut into agar slabs prepared as above. This elongated well was filled with 100 uL of viral antigen from the upper band in the sucrose-CsCl gradient, which had been dialyzed overnight against TE buffer. Electrophoresis was then carried out at 250 v for 2 h. The agar was then removed from the upper half of the plate by incision adjacent to the well and parallel to the direction of electrophoresis in the first dimension. The excised agar strip was replaced by an equal amount of the same agar containing 50 uL of partially purified immunoglobulin prepared against disrupted whole virus from the lower band in the sucrose-CsCl gradient. Electrophoresis was performed perpendicular to the first direction at 350 v for 180 min. The plates were then pressed, dried and stained as above.

O. Buoyant Density of Virus

The buoyant density of PPV was measured by correlation of the refractive index of individual fractions collected from equilibrium gradients with a standard reference table (Anderson and Anderson, 1968). Measurements were made at room temperature using an Abbe optical refractometer.

P. Preparation of Virion DNA

DNA was prepared most frequently by the method of Bourguignon et al (1976).

Purified virus was concentrated by dialysis against polyethylene glycol to a volume of 0.2 mL and layered on a 5-20% (w/v) alkaline sucrose gradient containing 300 mM NaOH, 700 mM NaCl and 0.15% (v/v) Sarkosyl. The preparation was centrifuged in a SW50 rotor at 170,000 g for 3.5 h in a Beckman L5-75 ultracentrifuge. Fractions were collected by bottom puncture and the optical density determined at a wavelength of 260 nm in a Gilford 250 spectrophotometer.

DNA was occasionally prepared by disruption of virions in 3 M guanidine hydrochloride as described by Shahrabadi et al (1977), or 0.75% SDS and 2.5 mM EDTA at 72.5°C for 90 min, followed by rapid cooling and precipitation of SDS and proteins with 1 M NaCl and centrifugation as described by May and May (1970).

Q. Agarose Gel Electrophoresis of PPV DNA

Agarose slab gels were prepared from 1% agarose in E buffer (40 mM Tris; 5 mM sodium acetate; 1 mM EDTA), using a Bio-Rad model 220 electrophoresis cell. Sample wells were formed directly in the top of the gel with a teflon comb. Bromophenol blue was added to samples as a tracking dye as was sufficient sucrose to produce at least a 10% solution. Samples were applied to the wells and electrophoresed in the same E buffer at 150 v until the tracking dye had migrated

4/5 of the gel length. Following electrophoresis, gels were either stained in ethidium bromide and immediately examined under ultraviolet illumination, or acridine orange followed by destaining in the appropriate buffer prior to examination with ultraviolet light. Human adenovirus serotype 5 (HAd5) DNA was run simultaneously as a double-stranded control for differential staining with acridine orange. Heat denatured DNA from phage ØX174 or HAd5 DNA cleaved by restriction enzyme Eco-R1 were occasionally electrophoresed simultaneously as controls.

R. SDS-Polyacrylamide Gel Electrophoresis of Virion Proteins

a) Preparation of Stacking and Resolving Gel. A modification of the procedure described by Laemmli (1970) was used for analysis of virion polypeptides. A 90 x 160 x 1.5 mm slab resolving gel consisting of 10% acrylamide (acrylamide:bisacrylamide ratio of 30:0.8) was prepared in a Bio-Rad model 220 apparatus and allowed to polymerize for at least 1 h. A 3% stacking gel was then prepared above this, containing ten sample slots formed with a teflon comb. The stacking gel was overlaid with electrode buffer and allowed to polymerize for 45 min prior to application of the samples.

b) Preparation of Samples. Purified virus from sucrose-CsCl gradients was drop dialyzed against 1% ammonium acetate for 45 min to remove salts (Marusyk and Sergeant, 1980). The sample was then diluted 1:5 with dissociating buffer containing 60 mM Tris, pH 6.8, 12.5% glycerol, 1.25% SDS, 1.25% mercaptoethanol and 0.001% bromophenol blue. The sample was heated in a boiling water bath for 2 min and then added to the sample well. HAd5 was treated similarly and added to the adjacent well as a control for polypeptide molecular weight determination (Marusyk and Cummings, 1978). Samples were electrophoresed at 30 milliamperes constant current until the marker dye was within 5 mm of the bottom of the gel, generally requiring 220 min. The slab gels were then stained in 0.2% Coomassie Brilliant Blue R prepared in methanol:glacial acetic acid:water (5:1:4) overnight. The following day the gel was destained until clear in the same solution without stain. The molecular weight of virion polypeptides was determined by comparison to the relative migration pattern of the known molecular weight polypeptides of the HAd5 control.

c) Densitometer Tracings of Stained Gels. The gels were sliced lengthwise into individual tracts, each containing the resolved polypeptide bands of each sample. The tracts were optically scanned at 590 nm wavelength in a Gilford spectrophotometer fitted with a linear transport scanner.

S. Thermal Denaturation of Purified Viral DNA

PPV DNA prepared by alkaline sucrose centrifugation was dialyzed overnight against 0.1 x saline sodium citrate (SSC) (1.5 M sodium chloride, 150 mM sodium citrate, pH 7.0) and diluted to approximately 0.1 optical density (OD) at 260 nm.

HAd5 DNA was prepared at the same concentration in the same buffer as a control. Both samples were heated to 100°C while being scanned for increases in optical density at 260 nm in a Pye-Unicam SP1800 recording spectrophotometer.

T. Electron Microscopy Studies

a) PPV Virions. Purified virus from CsCl gradients was drop-dialyzed against 1% ammonium acetate for 45 min and applied to formvar coated copper grids. Excess fluid was withdrawn with a filter paper and 1% sodium phosphotungstate was immediately added for 15 seconds and similarly withdrawn. Grids were allowed to dry and then examined in a Philips EM 300 and photographed with the 35 mm roll camera.

b) PPV DNA. Purified virion DNA prepared by alkaline sucrose sedimentation was examined according to the method of Davis et al (1971). The hyperphase spreading solution contained a final concentration of 100 mM Tris, 10 mM EDTA, pH 7.5, 40% formamide (v/v), 50 ug cyanogen bromide treated cytochrome C per mL and approximately 1 ug/mL PPV DNA (assuming 1 OD @ 260 nm = 36 ug/mL single stranded DNA; Sinsheimer, 1959). A small quantity of bacteriophage M13 DNA, RF1, the kind gift of Dr. A. Morgan was included as an internal control. This preparation was spread over a hypophase consisting of 10% formamide in 10 mM Tris, 1 mM EDTA, pH 7.5. The DNA was picked up on copper grids covered with carbon coated formvar films, that were then stained in an alcoholic solution of uranyl acetate and rotary shadowed with platinum-palladium. The grids were examined in a calibrated Philips EM 300 electron microscope and photographed.

The negatives were projected and traced at a final magnification of 70,000 x. Tracings of 15 circular M13 molecules and 168 PPV DNA molecules were measured with a map tracer. The approximate molecular weights of the various size classes were determined from the ratio of the projected lengths (M13 DNA = 2 x 10^6 daltons; Marvin and Hohn, 1969).

c) PPV-infected ST Cells. ST cells grown in Blake bottles were infected as previously described. At 72 h PI, when monolayers were just starting to show CPE, the infected cells were washed in 100 mM phosphate buffer, pH 7.2, and fixed with 3.5% glutaraldehyde prepared in the same buffer. After 5 min the cells were scraped from the bottle and centrifuged at low speed to form a pellet. The glutaraldehyde was removed and the cells were washed once in 1.7% sucrose in phosphate buffer and centrifuged again. The pellet was then post-fixed in 1% osmium tetroxide for 30 min. The cells were dehydrated in increasing concentrations of ethanol, followed by propylene oxide and finally embedded in Epon 812. After curing for 3 d in a 60°C oven, the specimens were trimmed and sectioned with a diamond knife and mounted on copper grids. The sections were stained with 5% uranyl acetate in methanol followed by lead citrate (Reynolds, 1963) and examined and photographed in a Philips EM 300 electron microscope.

U. Serological Survey

One hundred serum samples from market age swine in central Alberta were received from federal veterinarians. These sera were examined for evidence of previous PPV infection by the HI and CIE procedures described above.

V. Buffer Solutions

Buffer solutions used in this course of study were prepared according to the descriptions of Gomori (1955) or Tattersall et al (1976).

W. Manuscript Production

This thesis was drafted, edited and printed using the TEXTFORM program and the Amdahl 470V/6 computer of Computing Services, The University of Alberta.

III. RESULTS

A. Susceptibility of Various Cell Lines to PPV Infection

Various cell lines were examined both for evidence of contamination by subcytocidal levels of PPV (using the technique of Hallauer et al, 1971) and the production of CPE and viral hemagglutinin following attempted infection (Table 3). Only the ST cell line of porcine origin proved to be susceptible to infection by PPV. None of the human neoplastic cell lines from which Hallauer et al had readily isolated KBSH virus appeared able to support the growth of the ADRI-1 strain of PPV. Adenoviral pre-infection of KB cells did not vary the apparent non-permissive nature of this cell line, although an extensive study using various adenovirus serotypes and various MOI's was not performed. The alkaline glycine extraction procedure readily released viral hemagglutinin from infected ST cells with HA titres as high as 1:12800 obtained, varying with the volume of extraction buffer used.

B. Cytopathological and Morphological Alterations Produced by PPV in Cultured Swine Testicle Cells as Observed by Light and Electron Microscopy

Monolayers that were infected at 50% confluence progressed to a state of complete confluence during the first 48 h after infection with no evidence of degenerative change. Rapid degeneration progressed over the next 48 h

Table 3

The Production of Cytopathic Effect and Hemagglutinin by Attempted Porcine Parvovirus Infection of Cell Lines from Various Species

Cell Line	Uninoculated Control		Inoculated Principal	
	CPE	HA Titre	CPE	HA Titre
BHK 21	-	0	-	0
BSC 1	-	0	-	0
HeLa	-	0	-	0
HEp 2	-	0	-	0
KB	-	0	-	0
KB(HAd2 pre-infected)	++++*	0	++++*	0
LLC-RK 1	-	0	-	0
MRC 5	-	0	-	0
ST	-	0	+++	1024
Vero	-	0	-	0

- = no visible change
+ = minor degenerative changes, monolayer 25% affected
++ = moderate degenerative changes, monolayer 50% affected
+++ = severe degenerative changes, monolayer 75% affected
++++ = complete destruction of the monolayer

* typical adenovirus cytopathic effect

with the least confluent areas at the time of infection being affected first and the most confluent areas showing minimal change (Figure 3). The cytoplasm of infected cells began to retract, breaking cell-to-cell contact, and producing rounded cells with thin projecting membranous strands. Cells finally detached from the surface with progressively enlarging holes developing in the monolayer. The medium rapidly became acidic during the cellular degeneration.

Hematoxylin and eosin stained monolayers showed the presence of cytopathic alterations as early as 24 h PI. The earliest change observed was a fine basophilic prominence of the nuclear membrane. A more advanced stage of infection was indicated by a distinct darkening and thickening of the nuclear membrane with a barely discernible clear inner halo. The most advanced stage observed prior to cell lysis, demonstrated a prominently basophilic nuclear membrane, a wide clear intranuclear halo and a large, intensely basophilic, generally oval inclusion body enclosing the nucleoli (Figure 4). Although the number of cells demonstrating inclusions was variable and dependent on the multiplicity of infection, rarely did more than 25% of the cells show evidence of infection.

Electron microscopic examination of infected cells at 72 h PI demonstrated large numbers of granular amorphous inclusions, but few cells showed evidence of progressive assembly of complete virions. Severe degeneration of the

Figure 2

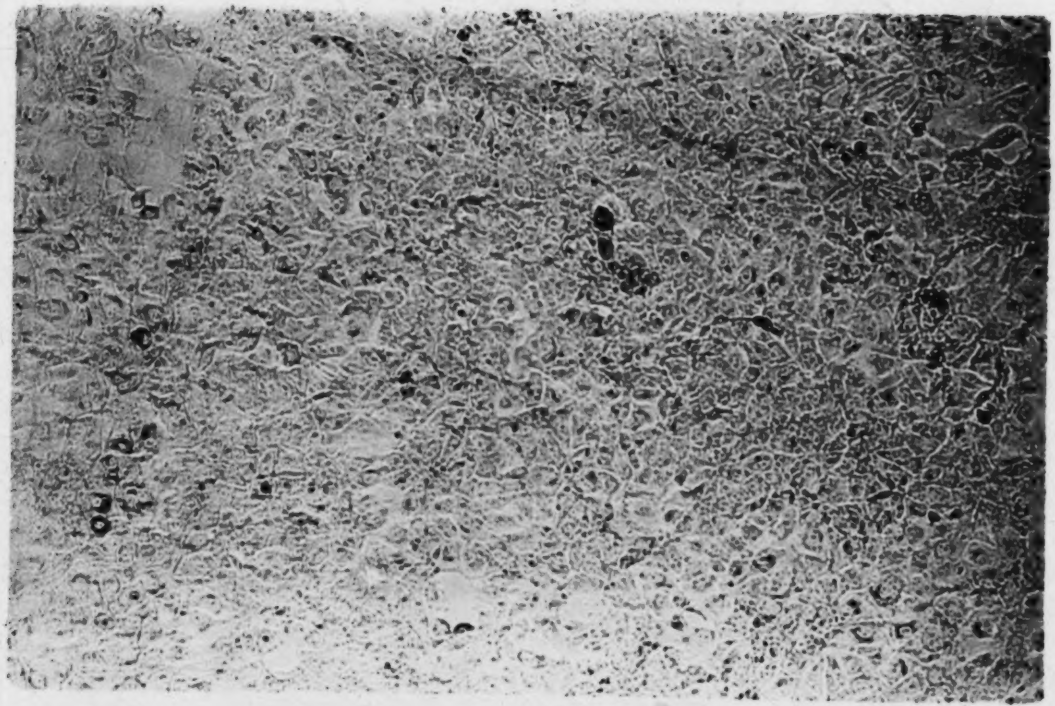
Unstained Swine Testicle Cell Monolayer

Swine testicle cells were grown in 60 mm diameter Petri dishes in growth medium supplemented with 10 mM HEPES at 37°C in a 5% CO_2 atmosphere. Photomicrographs were taken 4 d after the cells were seeded. Epithelial morphology is evident.
(Magnification: X 600)

Figure 3

Unstained Swine Testicle Cell Monolayer Infected with PPV

Subconfluent monolayers of ST cells in 60 mm diameter Petri dishes were infected 24 h after seeding with 1 mL of PPV (MOI 5 x 10^{-3} PFU/cell). Cells were examined and photographed 72 h PI. Notice the discrete rounding of cells and the large gaps that have developed in the monolayer.
(Magnification: X 600)



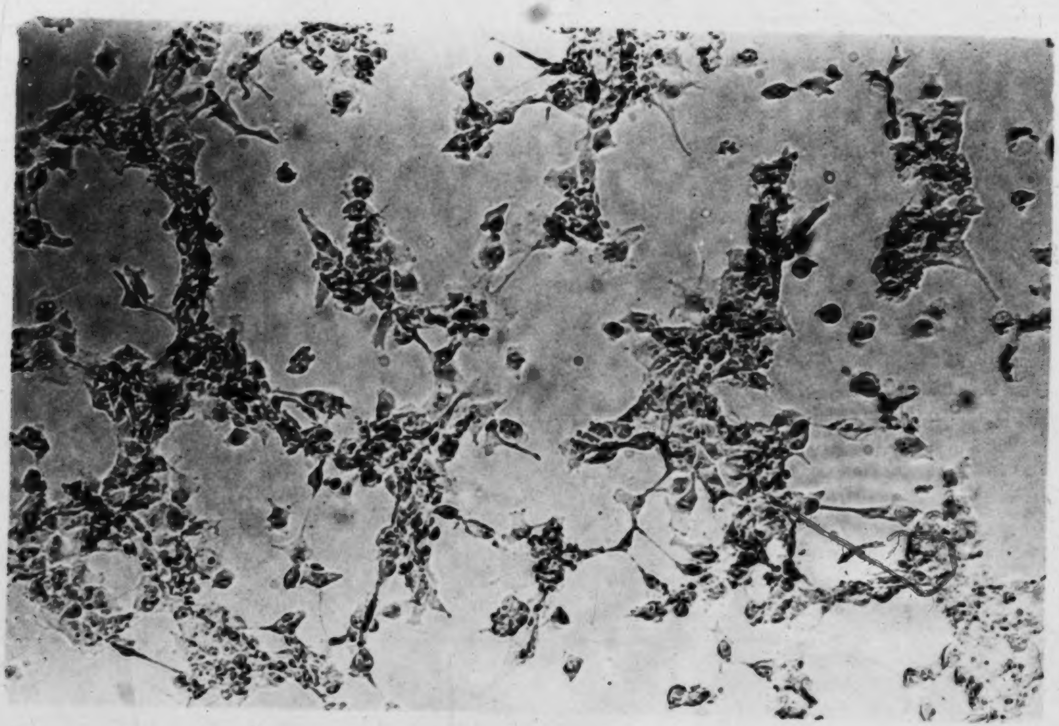


Figure 4

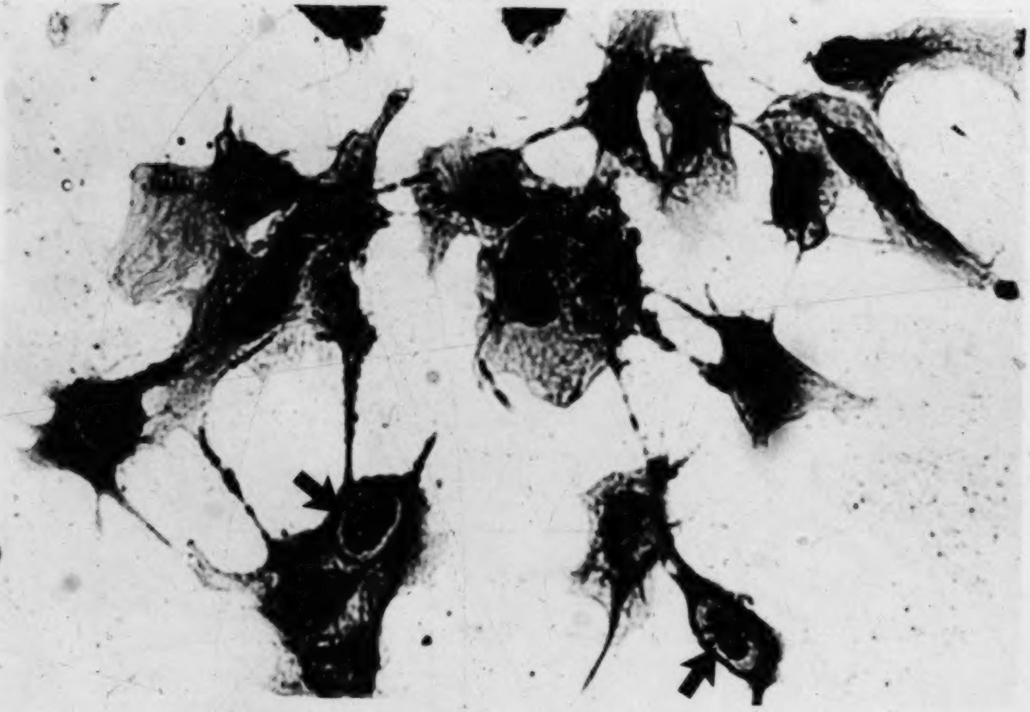
ST Cells Infected with PPV and Stained with Hematoxylin and Eosin

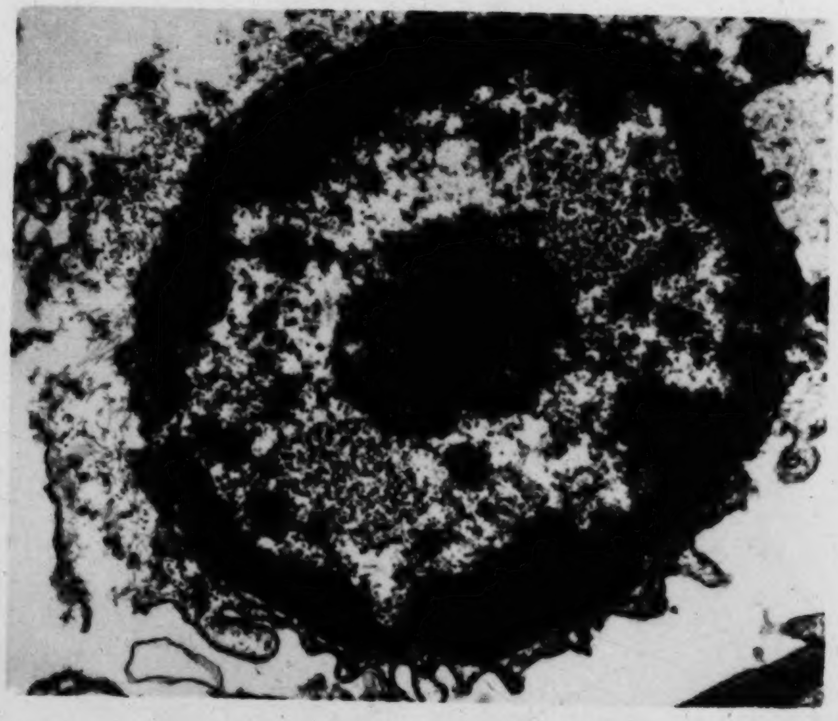
Subconfluent cultures of ST cells in Leighton tubes were inoculated with 1 mL of PPV (MOI 10^{-2} PFU/cell). Cells were fixed with Zenker's solution 48 h PI and stained with hematoxylin and eosin. Infected cells demonstrate large intranuclear inclusions surrounded by a clear halo and marginated chromatin (arrow).
(Magnification: X 2,500)

Figure 5

Electron Photomicrograph Section of ST Cells Infected with PPV

ST cells were infected with PPV (MOI 10^{-2} PFU/cell). 72 h PI cells from infected cultures were fixed, dehydrated, embedded, sectioned and stained as described in Materials and Methods. A large central, granular, intranuclear inclusion is evident. Numerous ribosomes can be seen immediately outside the degenerating nuclear membrane.
(Magnification: X 19,000)





nuclear membrane was evident at this time. A very thick band of amorphous homogeneous material lined the internal edge of the nuclear membrane. Large numbers of ribosomes could be observed immediately outside the nucleus in the cytoplasm (Figure 5). The inner halo observed by light microscopy was shown to contain large numbers of apparently empty and full particles. These often appeared to be in a string, presumably along euchromatin fibres (Singer and Rhode, 1978). The large granular inclusions could be seen to consist of massive aggregates of developing capsids (Figure 6). No evidence of lattice formation or capsomer structure was observed. Individual virions measured 20-22 nm diameter.

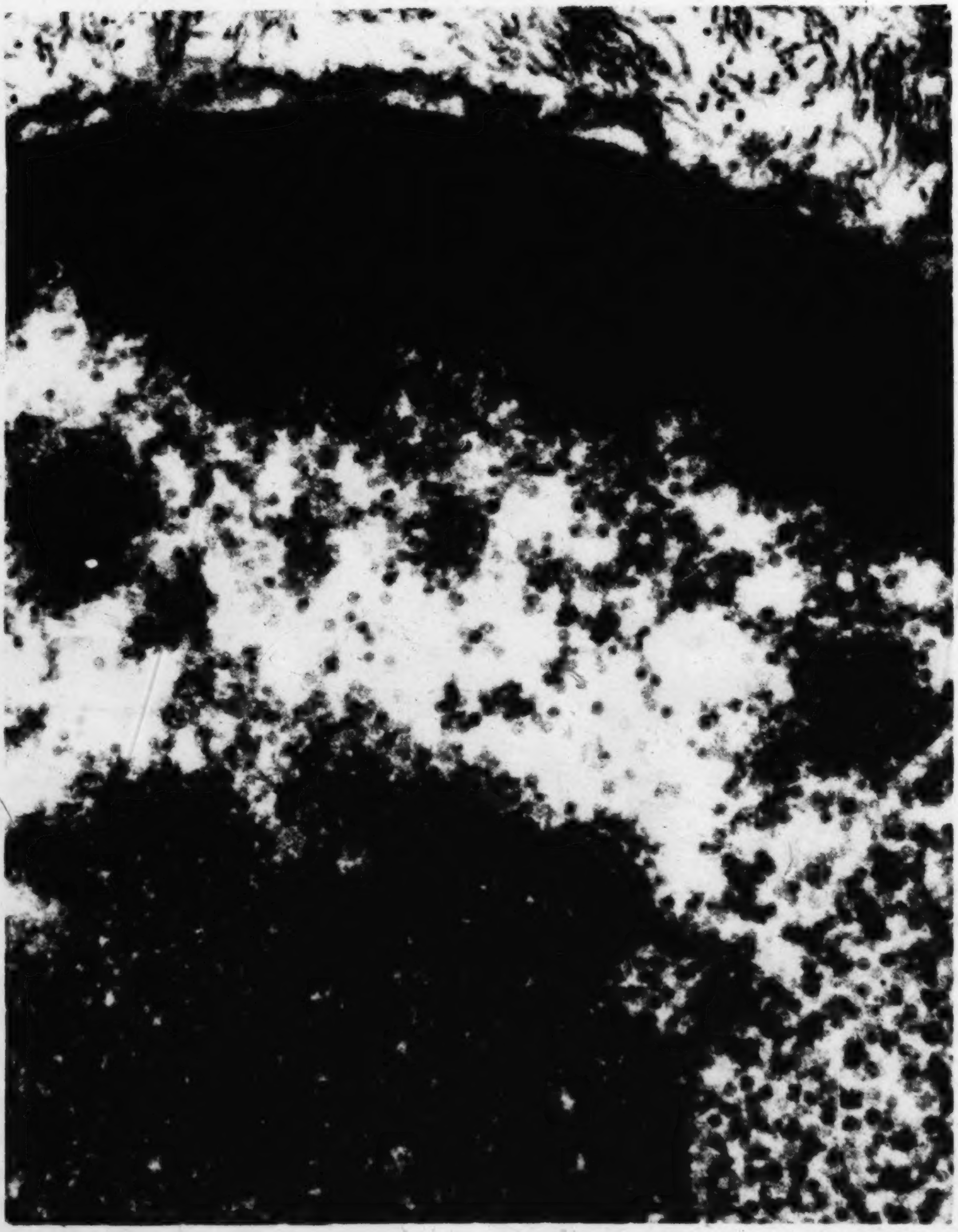
C. Purification and Concentration of PPV

Various methods have been described for the purification of parvoviruses. Several of these are suitable for producing a concentrated relatively pure virus preparation as judged by electron microscopy. The only procedure that proved adequately selective enough to produce essentially pure virus, as judged by SDS polyacrylamide gel electrophoresis (SDS-PAGE), is the minor modification of the procedure designed by Tattersall et al (1976; described earlier). The final two steps described by Tattersall et al, but not employed in this study, appear essential to remove minor quantities of residual nucleic acid and protein. This includes treatment with micrococcal nuclease followed by further sucrose sedimentation. The shortened Tattersall

Figure 6

Electron Photomicrograph Section of ST Cells Infected with PPV

The experiment was performed as described in Figure 5. Note the thick homogeneous, amorphous band lining the interior of the nuclear membrane. The granular central inclusion can be seen to consist of a large aggregate of developing viral capsids. Numerous full and empty virions can be seen in curvilinear arrays in the halo region.
(Magnification: X 98,000)



procedure as applied to PPV purification in this study tended to most routinely produce three distinct bands in the CsCl layer of the step gradient (Figure 7). A lower band with a sharp lower border and a somewhat diffuse upper border banding at a buoyant density of 1.40 gm/mL (Figure 8) was shown by SDS-PAGE and EM to consist of a heterogeneous population of empty and full, essentially pure, virions. A somewhat diffuse band appears higher in the gradient and from EM, HA and SDS-PAGE analysis consisted of a heterogeneous minor quantity of particles still partially attached to cellular debris. The upper band demonstrated a sharp lower border that barely entered the CsCl layer and merged imperceptively with cellular proteins, lipoproteins and lipids extending through the sucrose layer. Further analysis of the upper band by HA, electron microscopy, SDS-PAGE and immunoelectrophoresis suggested that it contained viral soluble components. Calculation of the efficiency of this purification procedure indicated that the lower gradient band contained 20% of total cellular hemagglutinin but only 10% of total infectivity (Table 4).

D. Infectivity Titration

Of the four methods examined for infectivity titration, only the TCID50 and plaque assays gave reliable objective results. The inclusion body assay and fluorescent focus assays routinely produced vague endpoints. Well developed intranuclear inclusions were readily detected by both

Figure 7

CsCl-Sucrose Step Gradient Centrifugation of Calcium Precipitated PPV

Virus was extracted and purified as described in Materials and Methods. A discrete lower band, diffuse middle band and broad upper band are evident.

Figure 8

Hemagglutination Profile of Serial Fractions from PPV CsCl-Sucrose Gradient Centrifugation.

Following centrifugation as described in Materials and Methods, 200 uL fractions were collected by bottom-puncture of centrifuge tubes. Fractions were assayed for hemagglutination and buoyant density and peak fractions were examined by EM.

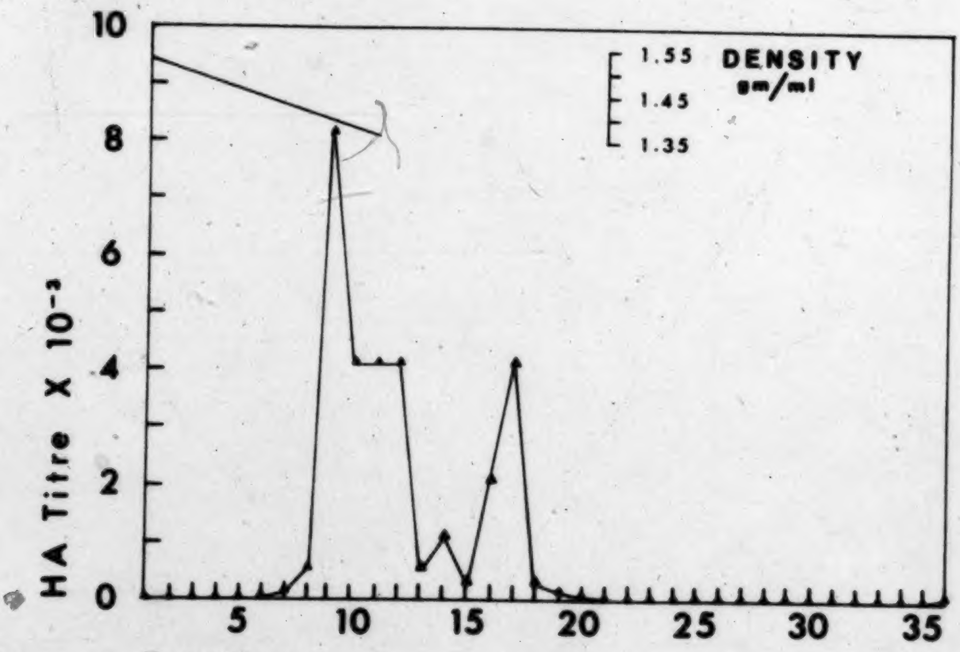

10
8
6
4
2
0
HA Titre X 10⁻³
1.55
1.45
1.35
DENSITY
gm/ml
5
10
15
20
25
30
35
Fraction

Table 4

Determination of the Efficiency of PPV Purification by the Modified Tattersall Procedure

	Cell Lysate	Purified Virus	Percent Recovery
Total Volume (ml)	20	1	
HA Titre (HAU/0.5 ml)	2048	8192	
Total HAU	8.19×10^5	1.6×10^5	20%
Infectivity Titre (PFU/0.2 ml)	10^5	2×10^5	
TOTAL INFECTIVITY (PFU)	10^7	10^6	10%

hematoxylin-eosin staining and fluorescent antibody staining. However as various developmental stages were present in each preparation, quantitation proved very difficult, despite an obvious dilution effect. Only intranuclear viral-specific fluorescence was prominent compared to uninfected control cells, in contrast to the findings of Mengeling (1972) who additionally reported early cytoplasmic fluorescence. The relatively high cytoplasmic background fluorescence present may have impaired detection of cytoplasmic viral antigen.

The TCID50 assay proved to be the most sensitive, but also the most tedious. Various types of commercial plastic culture tubes proved toxic to the ST cells, and individually washed glass tubes were necessary for successful application of this technique. A large quantity of culture medium was required (5 mL/tube) to provide for the substantial cell growth and acid production during the infection process.

Successful plaque production with PPV required strict adherence to procedural conditions and medium composition. 60 mm diameter petri dishes seeded with 3.5×10^5 cells each gave optimal cell density after an overnight incubation to allow cell attachment. At lower cell densities plaques could not be distinguished from gaps in the monolayer and at higher cell densities plaques were not visibly evident. Despite the presence of a carbon dioxide/bicarbonate buffer system, the inclusion of the organic buffer HEPES was found essential to buffer the large quantities of acid associated with significant cell growth under the agar. Failure to include HEPES buffer resulted in degeneration of the monolayer from toxicity prior to plaque formation. Very gentle application of the medium and PBS wash was necessary to prevent removal of significant numbers of cells from the sparsely seeded plates. The extremely thermosensitive nature of the ST cells necessitated critical control of the overlay medium temperature and rapid application of the overlay to obtain a uniform covering without heat necrosis of the monolayer. Maximum discrete plaque sizes of 1.5-3.0 mm were obtained at 5 d PI (Figure 9). Plaques were readily distinguished but did not possess an extremely sharp border. Plaques could be produced equally well from both crude and purified viral suspensions. A titre of 10^5 PFU/0.2 mL was found to be equivalent to 3.1 x 10^5 TCID50/0.2 mL.

E. Growth Curve

An illustration of the intracellular and extracellular growth curve of PPV in the ST cell line as measured by TCID50 and HA assays is shown in Figure 10.

As judged by TCID50 assay the virus latent period was 12-16 h. This was followed by a logarithmic increase in intracellular infectivity which reached a plateau at 36 h PI. Extracellular infectivity was not detected until 6 h after intracellular infectivity and followed a similar but more gradually sloping curve. A secondary late rise in infectivity, attributed in other studies to cleavage of the

Figure 9

Plaques Formed by PPV in ST Cells

Plaque assay of a crude viral inoculum was performed as described in Materials and Methods. The titration illustrated indicates an infectivity titre of 10^5 PFU/ 0.2 mL.

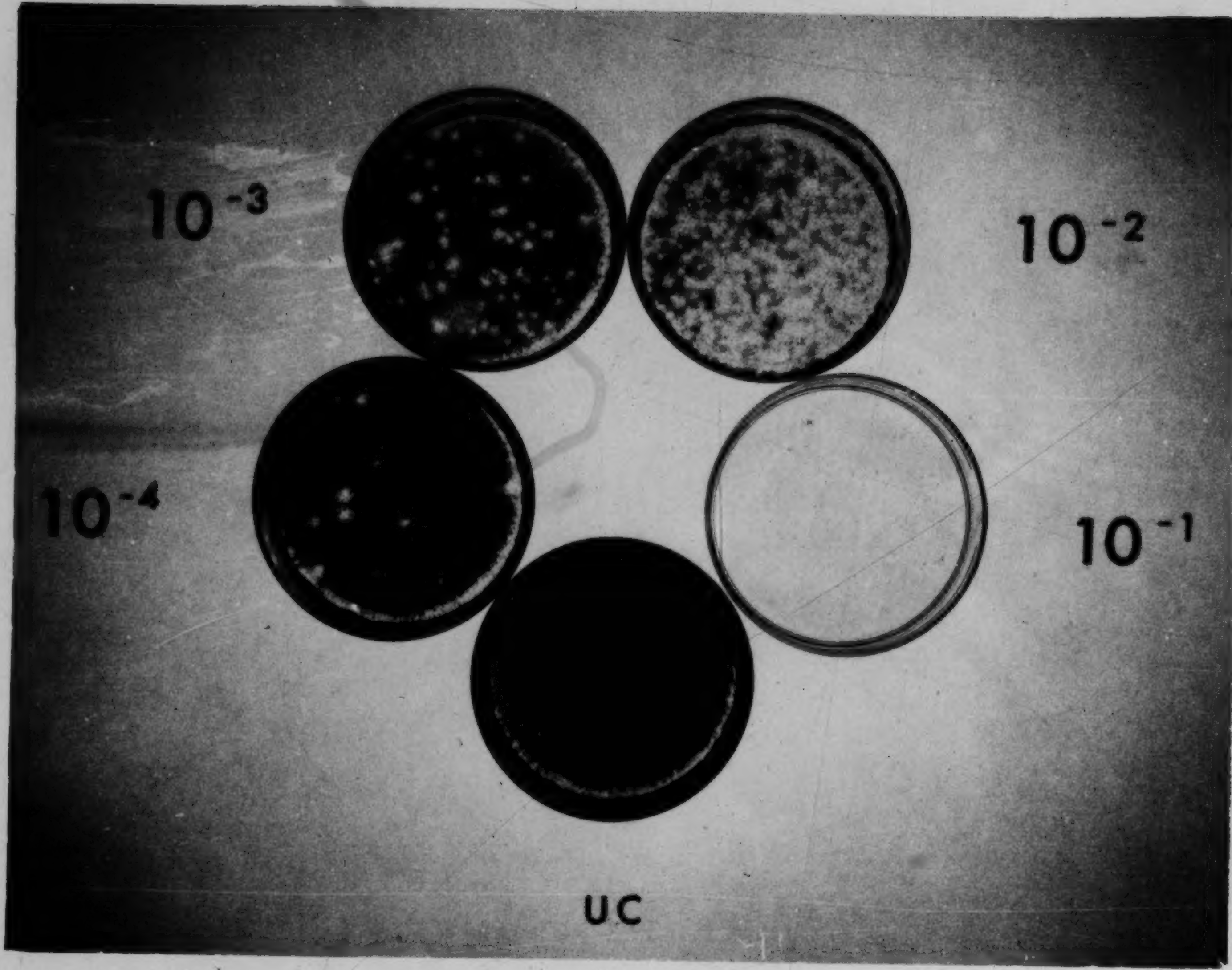
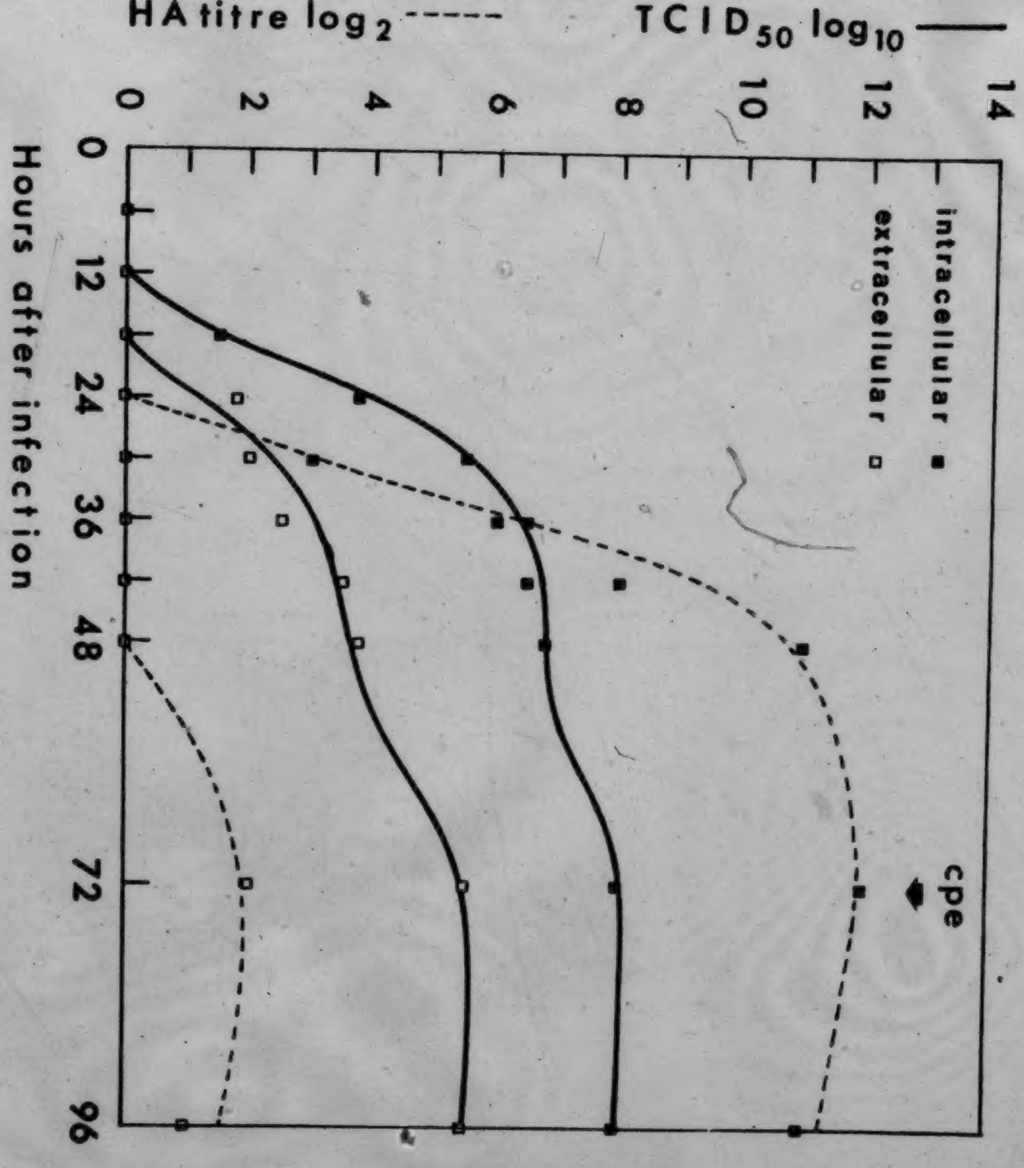

10^{-3}
10^{-2}
10^{-4}
10^{-1}
UC

Figure 10

One Step Growth Curve of PPV in ST Cells

Sparsely seeded, parasynchronized cultures of ST cells were infected at a MOI 10^{-3} PFU/cell. Unadsorbed virus was removed by several washes with PBS, and infection medium was added prior to incubation at 37°C. At various intervals until 96 h PI, cultures were examined for CPE and four cultures were randomly selected and pooled. Pooled samples were assayed for intracellular and extracellular viral hemagglutinin and infectivity as described in Materials and Methods.


TCID$_{50}$ log$_{10}$ ——
HA titre log$_2$ -----
0
2
4
6
8
10
12
14
0
12
24
36
48
72
96
Hours after infection
intracellular ■
extracellular □
cpe

B polypeptide to the C polypeptide, was noted between 48 and 72 h PI, as it has been with other parvoviral systems. Intracellular HA activity was not detectable until between 24 and 30 h PI, presumably due to the lower sensitivity of the assay. Increases in intracellular HA continued logarithmically until 48 h PI. Extracellular HA activity was not detectable until 48 to 72 h PI, when CPE was obvious. Apart from a slightly longer latent period, the shape of the growth curve resembles the single step growth curves reported for other parvoviruses (Tattersall, 1972; Singer and Rhode, 1978). This longer latent period may have been associated with the method of cell synchronization and the use of lower serum levels in the medium.

Comparison of the total infectivity (infectivity titre x volume) indicated that only approximately 10% of total infectivity was found in the extracellular fluid and 90% was associated with the cell pellet. Furthermore, comparison of the ratio of HA:infectivity for intracellular and extracellular samples revealed a five to tenfold difference indicating that HA is an extremely inaccurate way of estimating infectivity of crude viral preparations.

F. Hemagglutination

Hemagglutination tests were originally performed using 1% guinea pig erythrocytes. It was subsequently found that a 1% suspension of African green monkey erythrocytes, which are conveniently available in large quantity commercially without the need for terminal exsanguination of the donor, produced identical titres. The techniques of dilute alkaline buffer extraction and sonication each produced a one log difference in HA titre over cell suspensions freeze-thawed in neutral buffer. Relatively large volume preparations with HA titres greater than 4000 could be routinely prepared using these methods. Purified virus suspensions generally produced HA titres of 2000-8000.

G. Hemagglutination-Inhibition

Both the macro- and micro-titration methods were suitable for HI evaluation of sera. The macro-titration appeared to give clearer endpoints. Viral hemagglutinin prepared in either PPK or ST cells were equally reliable. Although plates were held for a final reading 18 h after addition of erythrocytes as described by Joo et al (1976b), little variation in HI titre was noted after 2 h incubation.

H. Countercurrent Immunoelectrophoresis

CIE proved to be an effective technique to identify the presence of soluble viral antigen when test samples were electrophoresed against specific antiserum, or the presence of PPV antibodies in test sera electrophoresed with a suitable known PPV antigen preparation. Tests generally gave positive reactions within 20 min, but electrophoresis was routinely continued for 1 h (Figure 11). Antigen preparations with HA titres of 4096 gave most satisfactorily

Figure 11

Countercurrent Immunoelectrophoresis of PPV Soluble Components

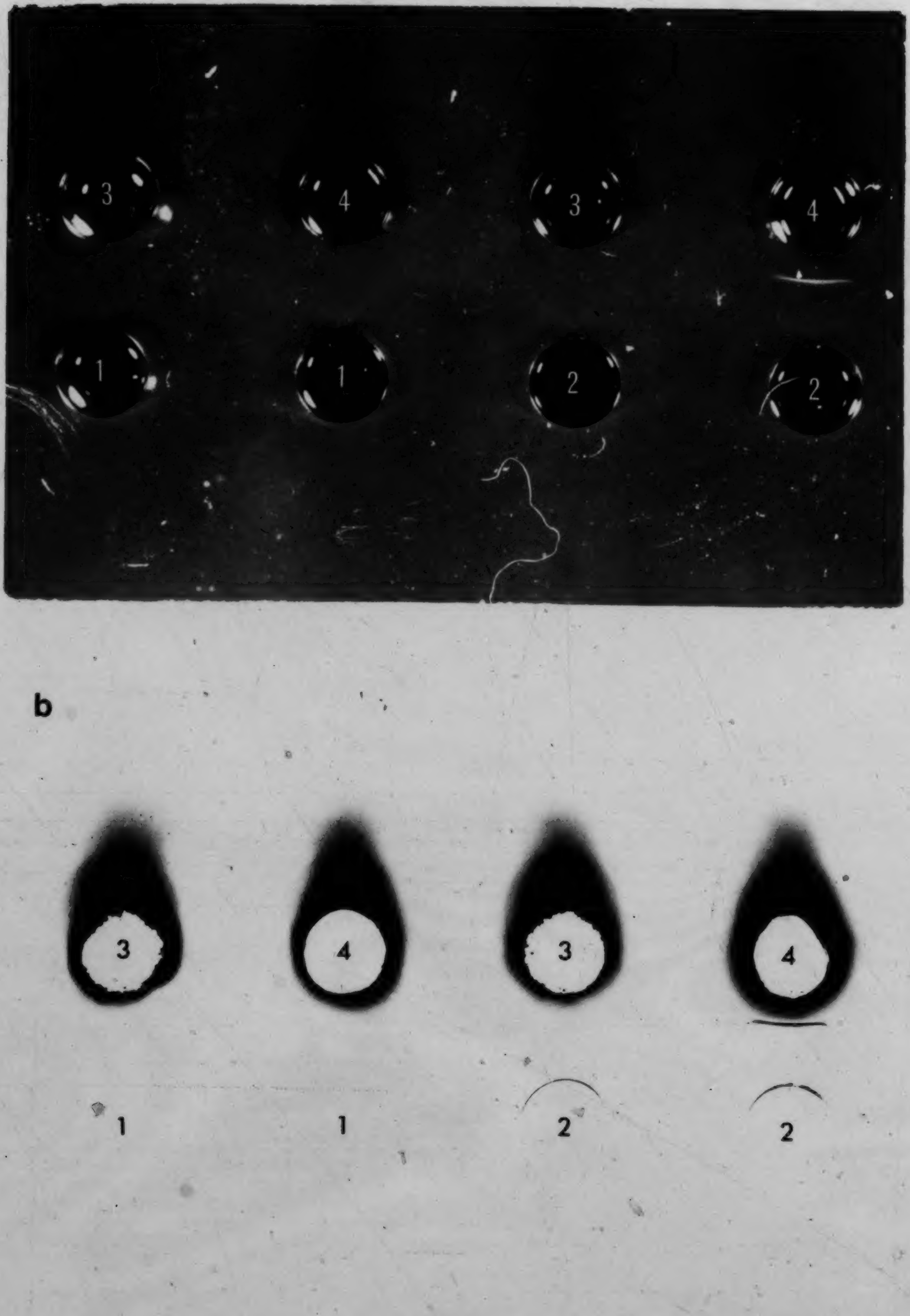
A suspension of PPV soluble components was prepared by alkaline glycine buffer and Freon extraction of PPV-infected ST cells. A control suspension was prepared by similar extraction of uninfected ST cells. 35 uL of antigen suspension was electrophoresed against a similar volume of test serum, as described in Materials and Methods. An obvious precipitin line could be readily detected immediately following the electrophoresis (a). Plates were routinely pressed, dried and stained for a permanent record (b).

1 control antigen preparation

2 PPV soluble antigen preparation

3 negative serum

4 positive serum


3
4
3
4
1
1
2
2
b
3
4
3
4
1
1
2
2

visible precipitates, while precipitates from antigen preparations with HA titres less than 512 were not readily apparent. Attempts to use fractions containing whole virus from CsCl-sucrose gradients were generally unsuccessful. Fractions from subsequent CsCl isopycnic gradients produced positive reactions throughout most of the gradient. Both diluted and undiluted serum could be used to produce obvious precipitin lines. The position of the precipitin line between the two wells was observed to vary with the HI titre of the serum and the HA titre of the antigen preparation. Positive reactions were consistently observed between antigens prepared in ST cells at various times throughout this study and antiserum prepared against whole virus or disrupted virus as well as antiserum received with the original viral isolate.

I. Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis was performed using aliquots from serially collected fractions of initial CsCl-sucrose step gradients, as well as peak viral fractions recentrifuged in CsCl equilibrium gradients, in an attempt to identify peak virus containing fractions. Initial step gradients rarely produced any significant rocket formation apart from fairly low, rounded blebs occuring with fractions from the upper region in the gradient suspected to contain PPV soluble components. Dark staining rims were present on the upper half of the wells which contained samples from fractions having peak HA titres, but no migration from the well was evident.

When fractions from subsequent equilibrium gradients were similarly examined a dramatic difference was observed. The fraction demonstrating a buoyant density of 1.44 gm/mL showed a large, well formed double rocket with similar minute rockets present in the two adjacent wells (Figure 12). It has been previously demonstrated that parvoviral particles of this buoyant density, the so-called heavy full particles, are readily disrupted by repeated CsCl centrifugation (Clinton and Hayashi, 1975). As numerous disrupted particles were evident upon electron microscopic examination of this fraction, it would seem that rocket formation was associated with migration of disrupted virion components and not complete virus.

J. Two-Dimensional Immunoelectrophoresis of PPV Soluble Components

Material collected from the upper band in sucrose-CsCl gradients was examined by CIE and rocket immunoelectrophoresis, prior to attempts at two dimensional immunoelectrophoresis, to assure adequacy of antigen. This material routinely gave a strong positive CIE reaction and produced short but obvious rockets, as opposed to purified virus which gave intense staining at the top of the wells, but no significant migration into the gel.

Two-dimensional immunoelectrophoresis against antiserum

Figure 12

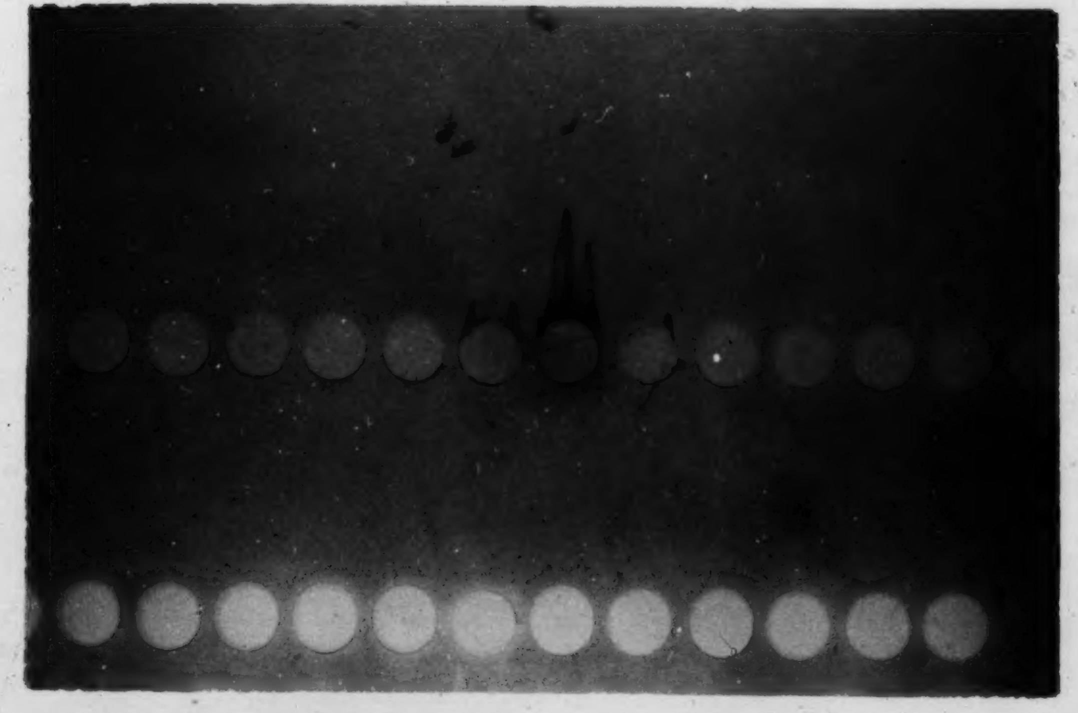
Rocket Immunelectrophoresis of Fractions Collected from CsCl Isopycnic Centrifugation of PPV

25 uL samples were added to wells cut in agarose slabs containing 50 uL anti-PPV immunoglobulin per 25 mL buffered agarose. Electrophoresis was performed at 250 v for 6 h, followed by pressing, drying and staining of the agarose slabs. A single fraction shows large prominent rockets with minor rockets present in the two adjacent wells.

Figure 13

Two-dimensional Immunoelectrophoresis of PPV Soluble Components

An aliquot from the upper band of CsCl-sucrose gradients was electrophoresed as described in Materials and Methods. The profile observed indicates the presence of two antigens with differing electrophoretic mobilities that share a common antigenic determinant.



to disrupted PPV routinely produced a pattern consisting of two closely migrating peaks joined by a line of identity (Figure 13). Attempts to produce greater peak height, resolution and separation by sample concentration, variation of antiserum concentration, voltage or electrophoresis time were unsuccessful. It is possible that this phenomenon was associated with residual cellular lipids bound to the soluble viral protein. Attempts to improve migration by freon or detergent treatment of the antigen were similarly unrewarding.

K. Ultraviolet Light Absorption Spectrum of Purified PPV

The absorption spectrum of PPV purified by the shortened Tattersall procedure is illustrated in Figure 14. The shape of the curve is very similar to that for another parvovirus, Minute Virus of Mice (MVM) (Tattersall et al, 1976). The E260/E280 ratio was calulated to be 1.45, slightly higher than the 1.38 value determined for MVM following treatment with micrococcal nuclease and further sucrose sedimentation in addition to the purification procedure used for PPV in this study (Tattersall et al,1976). The higher absorbance at 260 nm would most likely suggest residual contamination with small quantities of cellular nucleic acid or incompletely packaged replicative form viral DNA.

Figure 14

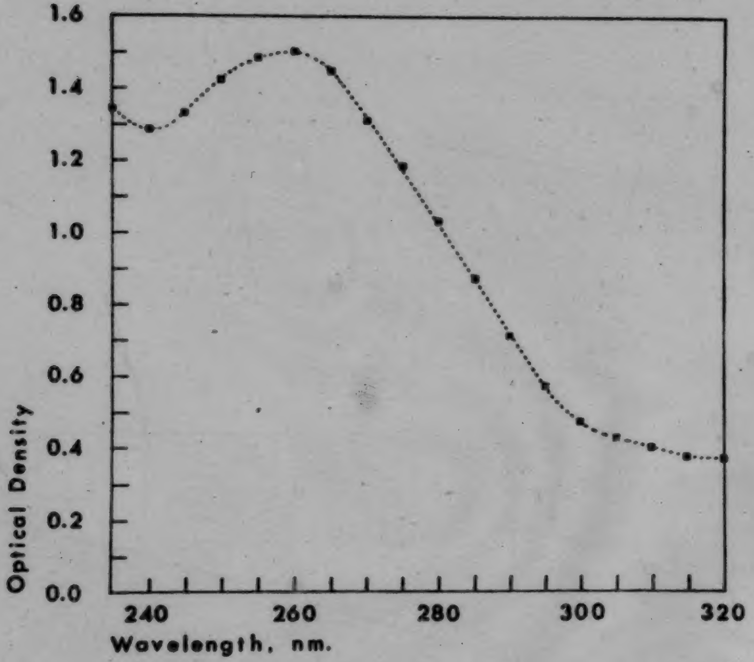
Ultraviolet Light Absorption Spectrum of PPV

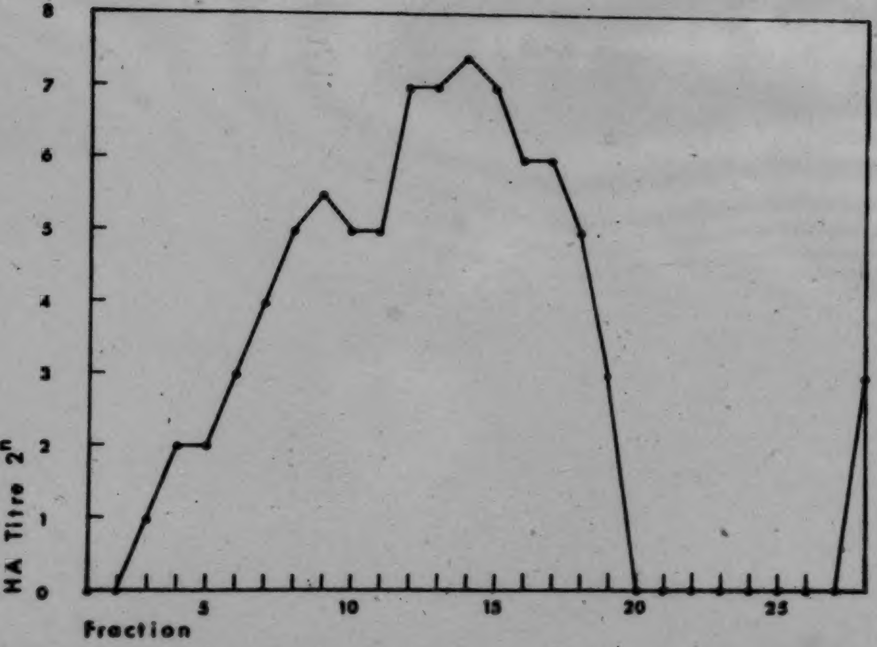
Purified PPV was obtained by the Tattersall procedure described in Materials and Methods. The ultraviolet light absorbance was measured in a Gilford 250 spectrophotometer. The E260/E280 ratio was 1.45.

Figure 15

Sucrose Sedimentation of PPV Virions

Purified PPV virions from CsCl isopycnic gradient were sedimented through a 5-20% neutral sucrose gradient as described in Materials and Methods. Fractions were collected by bottom-puncture of the centrifuge tubes and assayed for hemagglutination. A wide range of sedimentation rates is apparent, with two broad peaks of HA activity being detected.


Optical Density
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
240
260
280
300
320
Wavelength, nm.


HA Titre 2^n
8
7
6
5
4
3
2
1
0
5
10
15
20
25
Fraction

L. Sucrose Gradient Sedimentation of Purified PPV

Hemagglutinating activity could be detected over a very broad range in neutral sucrose gradients. As indicated in Figure 15, two peaks of hemagglutinin were evident with the slower sedimenting peak demonstrating the highest HA titre. The results of this analysis closely paralleled the findings of Muller et al (1978) during studies on the production of defective particles of parvovirus Lu III following multiple undiluted passage of virus. Such a procedure had been performed early in this study of PPV, without expectation of this consequence. The presence of a very heterogeneous population of particles, with respect to their sedimentation co-efficients, was suggested by this profile as well as a predominance of more slowly sedimenting defective particles within this population.

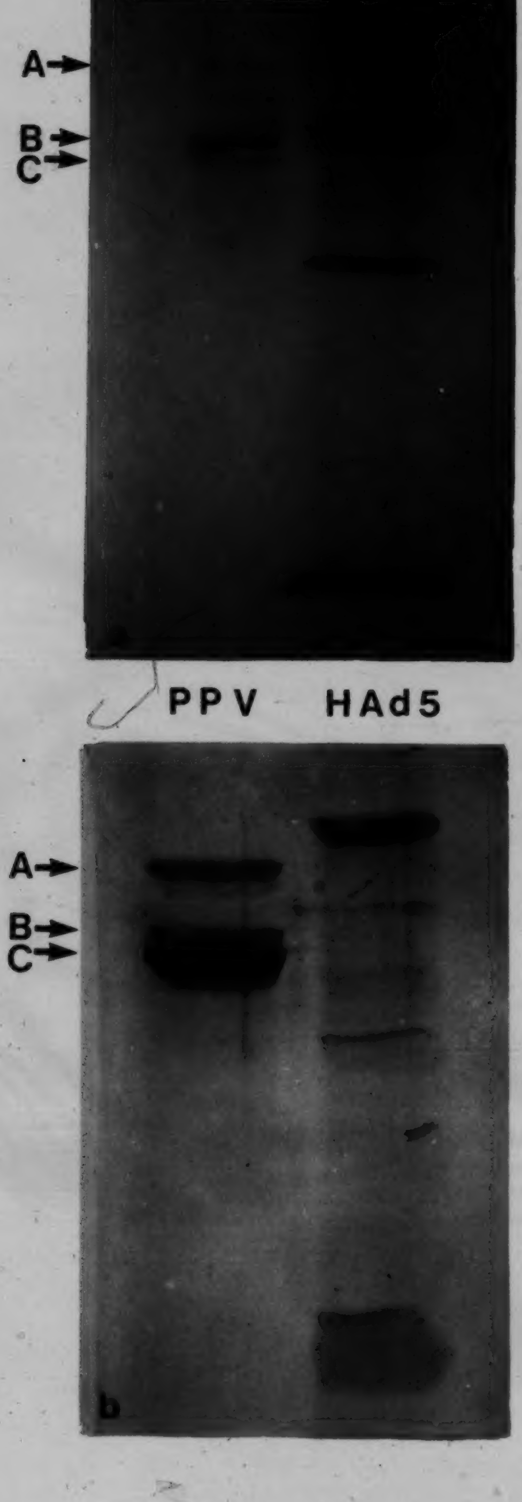
M. SDS-Polyacrylamide Gel Electrophoresis of Purified PPV

Disruption of purified virus into component polypeptides and subsequent separation according to molecular weight by polyacrylamide gel electrophoresis was performed (Figure 16). Three polypeptide species designated A, B and C in order of decreasing molecular weight (following the precedent of Tattersall et al, 1976) were demonstrated. By comparison of the relative migration patterns of the PPV polypeptides to those of HAd5, for which published molecular weights are available (Marusyk and Cummings, 1978), an accurate estimate of the molecular

Figure 16

SDS-Polyacrylamide Gel Electrophoresis of PPV Polypeptides

PPV purified as described by Tattersall et al (1976) in the presence of a proteolytic enzyme inhibitor was analysed by the SDS-PAGE technique as described in Materials and Methods (a). HAd5 was similarly analyzed as a control. Three parvoviral polypeptides are indicated by this analysis. The B polypeptide is observed to be the predominant species. PPV purified as described by Bourgignon et al (1976) in the presence of the proteolytic enzyme trypsin was similarly analyzed (b). Again three polypeptides are observed. However, the faster migrating C polypeptide is predominant.


A
B
C
PPV
HAd5
A
B
C
b

weight of the three PPV polypeptides was obtained. As determined from densitometer tracings (Figure 17), the molecular weights are 87,000 (polypeptide A), 69,000 (polypeptide B) and 66,000 (polypeptide C).

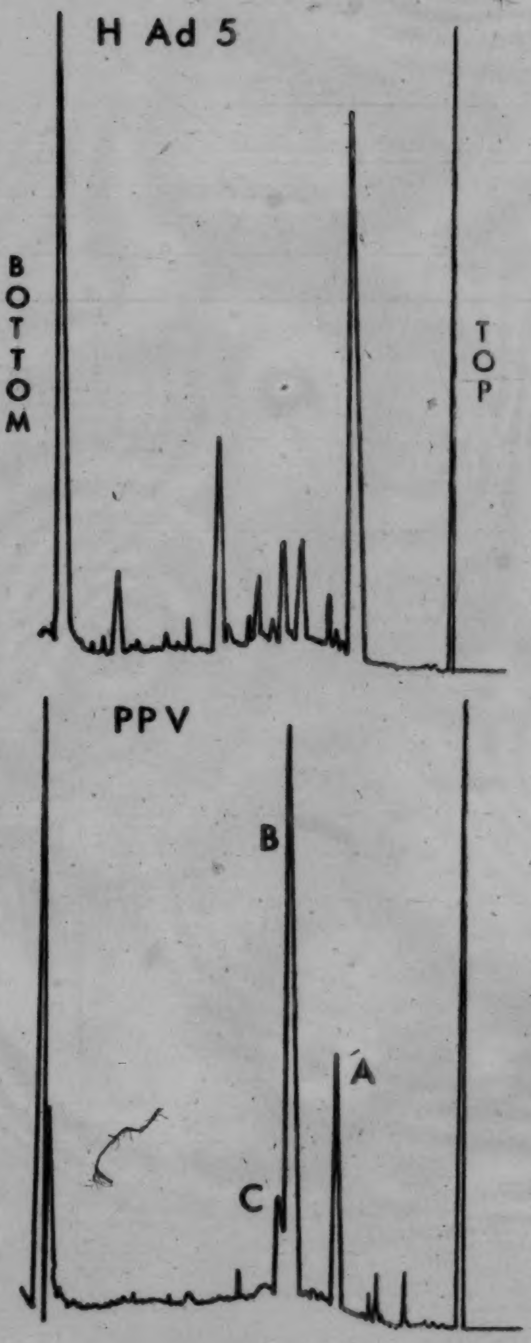
Considerable variation in the proportion of each polypeptide present and the presence of additional polypeptide species was found to depend on the method of purification used and the time of virus harvest. Purification methods not employing a calcium precipitation step routinely contained various arrays of additional polypeptides. As virus preparations not containing these polypeptides were completely infectious, it is assumed that these were contaminant cellular polypeptides. Most prominent in these additional polypeptides were a 72,500 dalton species and a 55,000 dalton species. Polypeptide A comprised 10-20% of the total protein in all preparations, as estimated from the area under the curve on densitometer tracings. Polypeptide B was the predominant species and generally accounted for 60-80% of the total protein. Polypeptide C most frequently appeared as 20% or less of the total protein. When trypsin was used in purification (Bourgignon et al, 1976), a significant increase in the proportion of polypeptide C was evident. Trypsin has been reported to cause cleavage of the B polypeptide of heavy full particles to a C polypeptide (Tattersall et al, 1976). Preparations harvested before CPE was evident occasionally produced only the A and B polypeptides. The in vivo

Figure 17

Densitometer Tracing of PPV Polypeptides Separated by SDS-Page

Stained polyacrylamide gels containing PPV polypeptides separated by SDS-PAGE were scanned at 590 nm wavelength in a Gilford spectrophotometer with a linear transport scanner. The molecular weights of the separated polypeptides were determined by comparison to the relative migration of HAd5 polypeptides.

The molecular weights determined by this analysis were:
Polypeptide A - 87,000
Polypeptide B - 69,000
Polypeptide C - 66,000


H Ad 5
BOTTOM
TOP
PPV
B
A
C

conversion of polypeptide B to polypeptide C is considered to occur at a late stage in infection (Tattersall, 1978a) and to be associated with the late secondary rise in infectivity noted in this (Figure 10) and other studies (Singer and Rhode, 1977b).

N. Alkaline Sucrose Gradient Sedimentation of PPV DNA

Sedimentation of purified PPV into an alkaline sucrose gradient produced the most suitable DNA preparation for electron microscopy. A small amount of rapidly sedimenting, presumably cellular, DNA could be detected by ultraviolet absorption measurement at 260 nm (Figure 18). Under the conditions used, the PPV DNA sedimented as a single band with a steep leading face and a broad trailing edge.

O. Thermal Denaturation Curve

Figure 19 illustrates the results of two thermal denaturation experiments. The curve associated with the change in optical density at 260 nm wavelength for PPV DNA during heating to 100°C shows only a minor terminal increase. In sharp contrast, the curve produced with HAd5, a known double-stranded virus, demonstrates a substantial increase in optical density as the sample is heated. On the basis of these experiments the DNA of PPV is indicated to exist in a single stranded configuration.

Figure 18

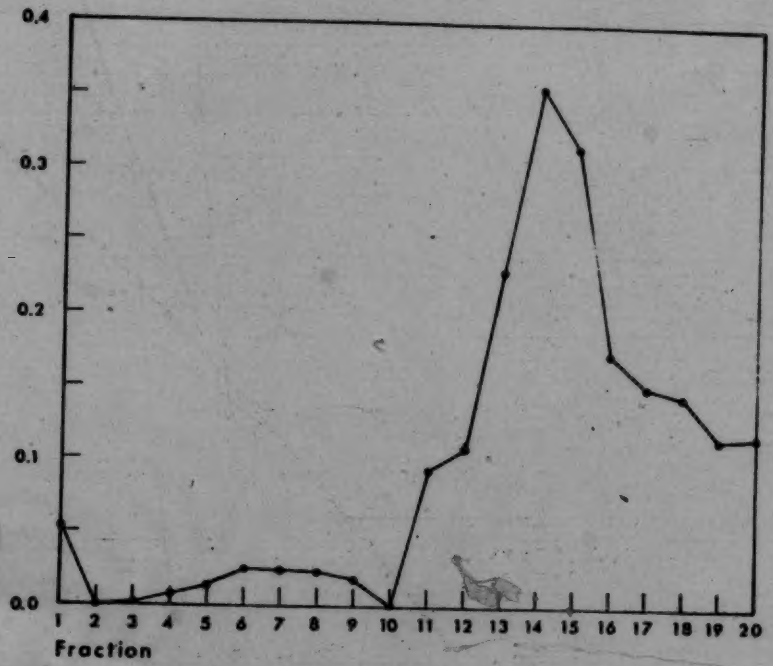
Alkaline Sucrose Sedimentation of PPV DNA

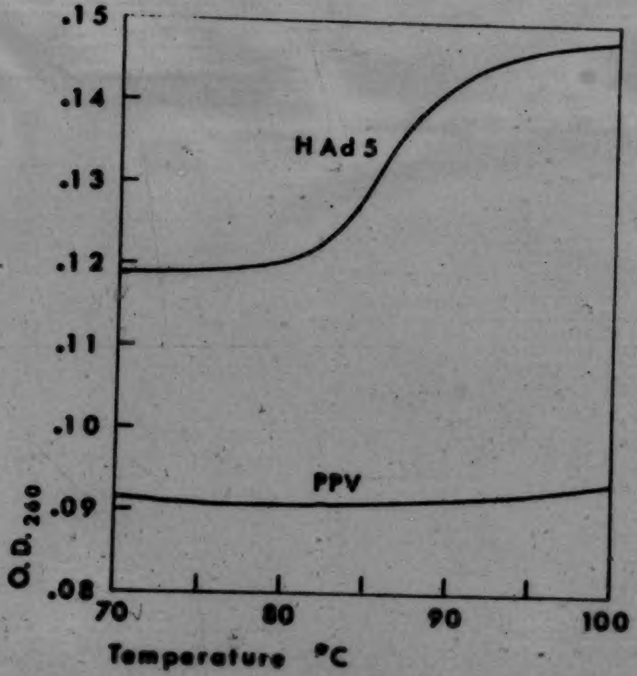
Purified PPV was layered on a 5-20% alkaline sucrose gradient in a SW 50 rotor and centrifuged for 3.5 h at 170,00 g as described in Materials and Methods. 200 uL fractions were collected by bottom-puncture of the centrifuge tube and analyzed for ultraviolet light absorption at 260 nm wavelength.

Figure 19

Thermal Denaturation Curve of PPV DNA

Samples of PPV DNA and HAd5 DNA were diluted to 0.1 O.D. @ 260 nm with 0.1 x SSC. The samples were heated to 100°C in a Pye-Unicam 1800 spectrophotometer while the optical density was recorded. A distinct sigmoid curve increase is apparent with the HAd5 sample, characteristic of a double-stranded configuration. The PPV DNA demonstrated minimal change during heating to 100°C, indicating a single-stranded configuration.


0.4
0.3
0.2
0.1
0.0
O.D.$_{260}$
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Fraction


.15
.14
.13
.12
.11
.10
.09
.08
O.D.$_{260}$
H Ad 5
PPV
70
80
90
100
Temperature °C

P. Agarose Gel Electrophoresis of PPV DNA

A broad continuous band was repeatedly produced by agarose gel electrophoresis of PPV DNA. This was in sharp contrast to the discrete band produced by the adenovirus DNA control sample. The parvoviral DNA had the same somewhat diffuse appearance regardless of the method used to separate viral DNA and protein, suggesting that the broadness of the band was truly an indication of the heterogeneous population of DNA lengths in the sample and not an atrifact. When parvoviral DNA was electrophoresed with a control consisting of restriction enzyme Eco-R1 generated fragments of HAd5, the upper limit of the PPV DNA band migrated slightly ahead of the smallest restriction fragment. Although this would suggest that the complete PPV genome is slightly less than 1.7 million daltons (Mulder et al, 1974), a direct comparison of single- and double-stranded molecules is inconclusive. More suitable single-stranded controls were unavailable. The differential staining properties of the acridine orange dramatically distinguished between the intense green fluorescence of the double-stranded adenovirus DNA and the weaker flame red fluorescence of the PPV DNA, again indicative of a single stranded configuration. Furthermore, ribonuclease treatment did not alter the appearance of the PPV nucleic acid band. Unfortunately due to the diffuse nature of the PPV band and the weak red fluorescence, satisfactory photographs were not obtained.

Q. Electron Microscopic Observation of Virion Morphology

Virions purified by the shortened Tattersall procedure demonstrated the least evidence of residual contaminating cellular debris (Figure 20). Virions from such preparations revealed a generally spherical morphology. Capsomer structure was too indistinct to allow description. Particles frequently clumped to form a hexagonal lattice, suggestive of icosahedral symmetry. Apparently intact virions whose centre had been penetrated with stain, presumably empty particles, comprised approximately 25% of most preparations. A large number of damaged particles were routinely seen in various stages of degradation into an inner core (approximately 12-14 nm in diameter) and an outer shell. Individual virions varied in size from 20-24 nm in diameter with an average of 22 nm.

R. Electron Microscopic Observation of Purified PPV DNA

DNA prepared by alkaline sucrose gradient centrifugation proved most suitable for electron microscopy. Residual protein in preparations disrupted in guanidine hydrochloride resulted in clumping of the released DNA.

Purified DNA spread by the formamide technique exhibited a profoundly heterogeneous distribution of lengths (Figure 21). The molecules were all linear and gave the appearance of being single-stranded in comparison to the double-stranded internal control molecules. They ranged in measured length from 0.15 to 1.05 microns (u) as judged by

Figure 20

Electron Photomicrograph of Purified PPV Virions

Purified PPV from CsCl-sucrose gradients was drop-dialyzed against 1% ammonium acetate, applied to formvar-coated copper grids and stained with 1% sodium phosphotungstate. The average diameter of the virions observed was 22nm. Numerous full and empty virions can be observed as well as some particles in the process of disrupting into an inner core and an outer shell (arrow).

(Magnification: X 165,000)

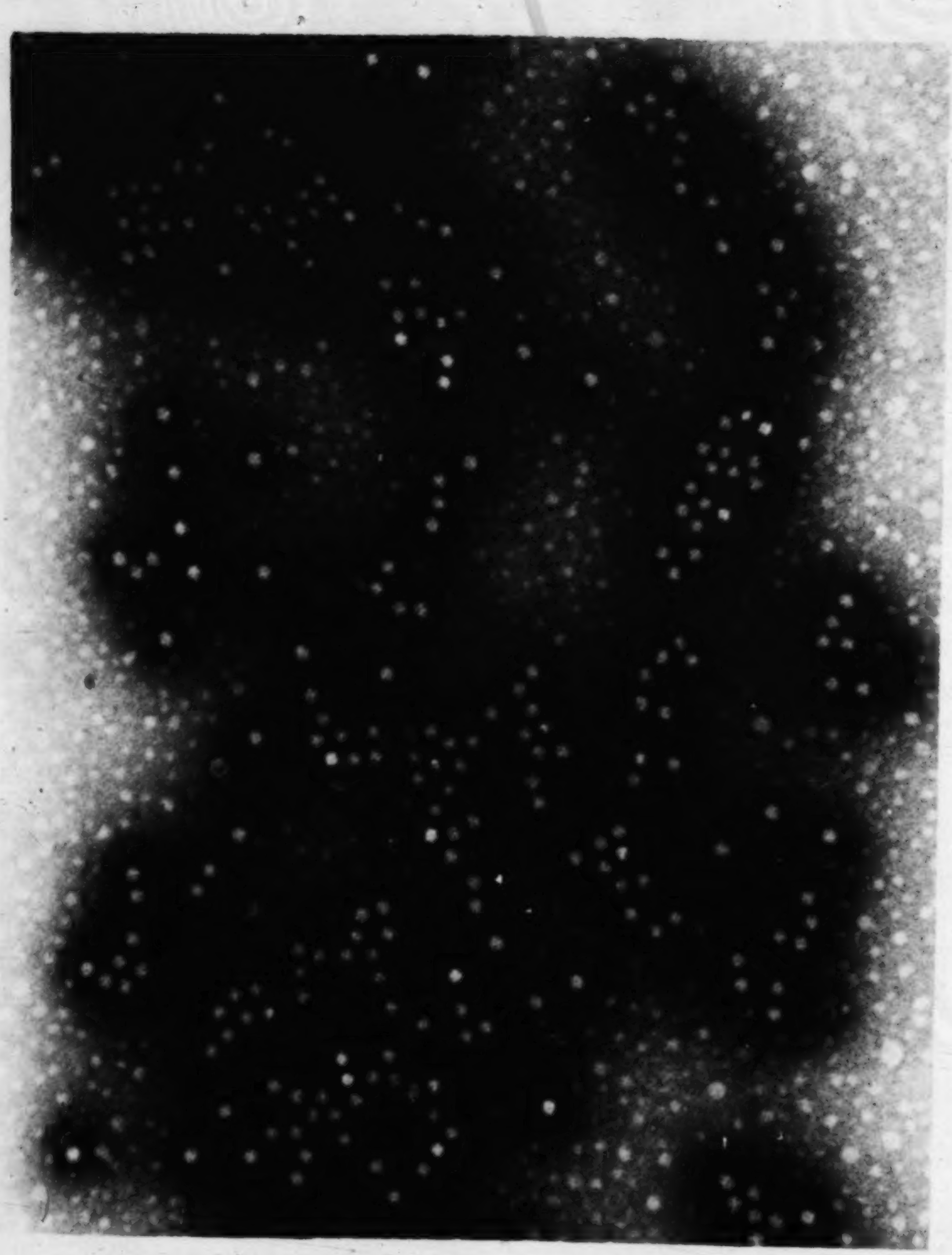


Figure 21

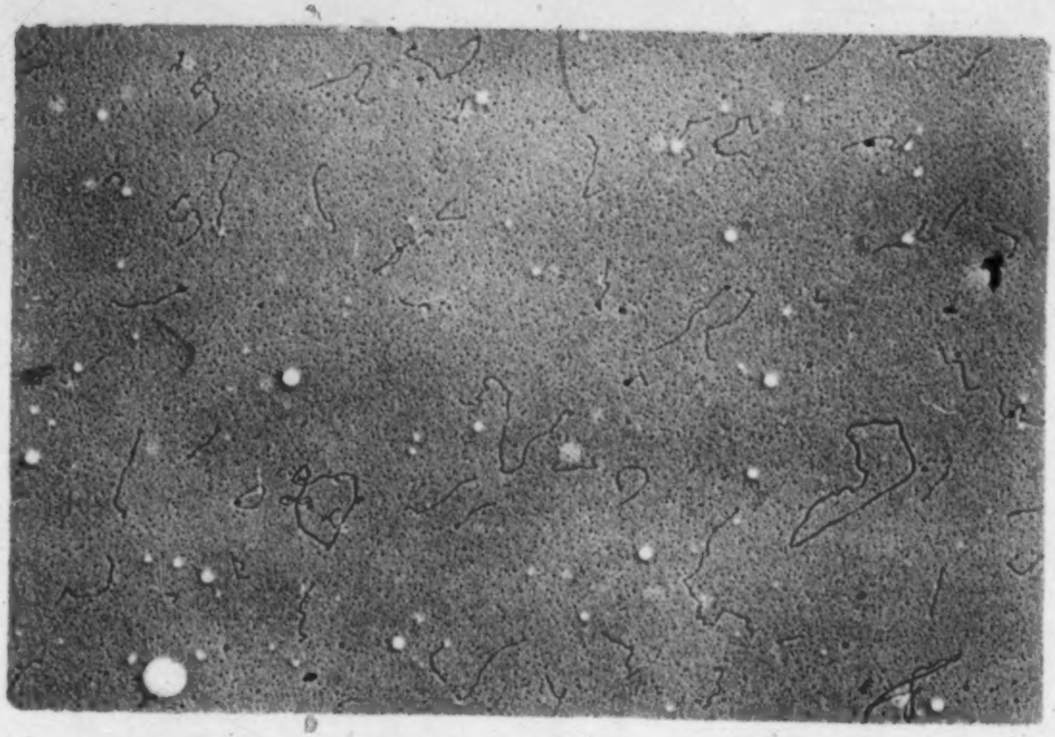
Electron Photomicrograph of PPV DNA

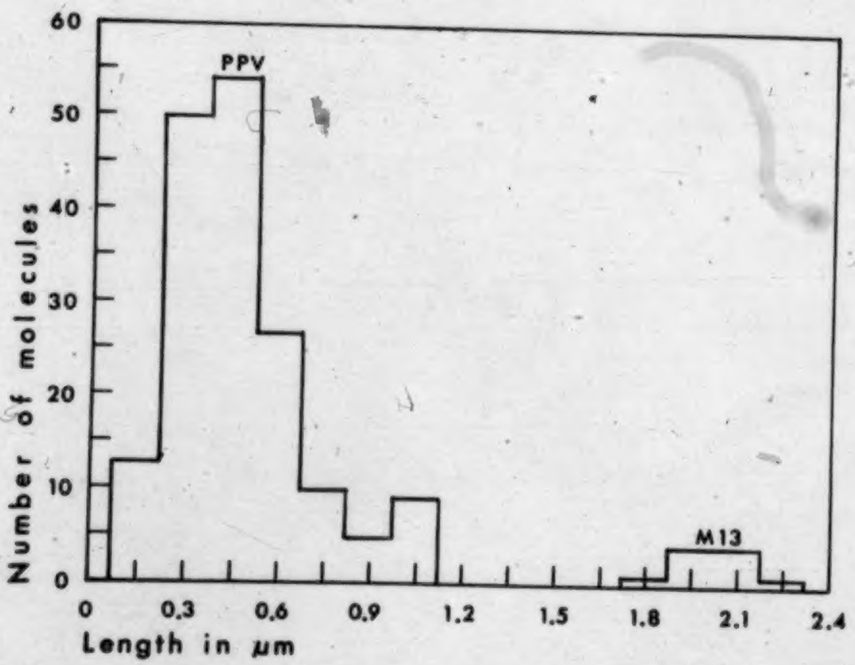
PPV DNA was isolated by alkaline sucrose gradient centrifugation. The DNA preparation was mixed with a small quantity of circular phage M13 DNA and spread by the formamide technique. The spread DNA was picked up on carbon coated copper grids, stained with uranyl acetate and shadowed with platinum-palladium. The thicker double-stranded circular phage DNA is readily distinguishable from the linear, single-stranded PPV DNA. Notice the heterogeneous distribution of PPV DNA lengths present. (Magnification: X 34,000)

Figure 22

Histogram of PPV DNA Length Distribution

168 PPV DNA molecules and 15 phage M13 DNA molecules were measured from photographic enlargements using a map tracer. The size and molecular weight of the PPV DNA molecules were estimated by comparison to the M13 DNA internal standard. The complete genome of PPV by this analysis is estimated to be 1.05×10^6 daltons.




PPV
M13
Number of molecules
0
10
20
30
40
50
60
Length in µm
0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
2.4

comparison to the control molecules and calibration with the microscope magnification factor. Only 9 of the 168 molecules measured demonstrated the maximum size, assumed to be complete genome length (Figure 22). The most frequently occuring size class was 0.45 u or approximately 43% genome length. By comparison to the control molecule length, the complete PPV genome would appear to be 1.05 million daltons, somewhat short for the quoted range of 1.5-2.2 million daltons. As single-stranded parvoviral DNA does not spread as completely as double-stranded DNA (Singer and Rhode, 1977a), even in the presence of formamide, it is believed that this deficiency is an artifactual result of using a double-stranded internal control. A suitable single-stranded internal control unfortunately was unavailable for use.

S. Serological Survey

The results of a serological survey conducted to evaluate the frequency of PPV infection in swine in Alberta as well as the reliability of the CIE test described above are presented in Table 5.

One hundred serum samples collected from market age swine from four different abattoirs in the Edmonton-Red Deer area were supplied for analysis by federal veterinarians. As high titres were expected, sera were first examined by the macro-titre method in the range of 800 to 25600. Samples giving negative titres in this range were further examined in the range of 8-1024 by the micro-titre test. Upon arrival

Table 5

A Serological Survey of Sera from Swine in Central Alberta to Detect Antibodies to PPV

	HI		CIE	
Results	No.	%	No.	%
Positive	68/89	76	67/89	75
Negative	21/89	24	22/89	25
Titre Range	1:64 - 1:12800		N/A	

eleven samples were judged unsuitable for testing due to deterioration during shipping. Sixty-eight of eighty-nine samples (76%) demonstrated HI titres to PPV. Titres ranged from 1:64 to 1:12800 with 63 sera having titres of 1:800 or greater. These values fell in the midrange of findings for similar surveys done in several countries, as reviewed by Joo and Johnson (1976).

The sera tested for HI antibodies to PPV were also examined by CIE as described in Materials and Methods. Apart from one sample giving a positive HI test and negative CIE test, all results correlated completely . Precipitin lines were generally visible after electrophoresis for 30 min without staining but electrophoresis was routinely continued for 1 h and the plates were pressed, dried and stained to obtain maximum sensitivity. Unstained plates often appeared to demonstrate two closely spaced fine lines. Upon pressing and staining, however, only a single line was visible. An antigen preparation containing 4096 HA units per 0.05 mL gave the most plainly visible line, conveniently positioned midway between the wells. CIE using the uninfected control cell extract did not produce a precipitin line with any of the test sera.

IV. DISCUSSION

The lack of conveniently suitable methods for the large scale growth, purification and objective quantitation of PPV has severely limited the complete characterization of this virus in comparison to other parvoviruses described to date. Similarly, the lack of reliable diagnostic reagents and the inavailability of standardized vaccines has impaired clinico-pathological studies into the relative significance of PPV in porcine reproductive failure and the value of biological prophylaxis. The various observations recorded throughout this study have been examined in an attempt to confirm the classification of PPV as a parvovirus and to elucidate some of its unique properties with respect to growth in cultured cells.

The primary prerequisite to such a study was the procurement of a susceptible cell line. In addition to the successful identification of a suitable cell line for the propagation of PPV during the screening procedure, data was obtained which supported the strict host species specificity demonstrated by PPV in other studies (Mayr et al, 1968; Cartwright et al, 1969). The species specificity observed is in distinct contrast to characteristics of the KBSH virus strain (Hallauer et al, 1971) which could be readily recovered from several human cell lines as a presumed contaminant and similarly propagated in these cell lines. Furthermore, the lack of evidence to suggest parvovirus contamination in any of the cell lines examined during the present study, despite their diverse geographical origins and high passage levels, supports the supposition by Hallauer et al (1971) that the high contamination level observed (27 of 41 cell strains) with the KBSH serotype was a localized phenomenon. It seems somewhat remiss that Hallauer et al did not report such simple procedures as the range of species erythrocytes agglutinated by KBSH, or the susceptibility of porcine cell lines to KBSH infection. Obviously, a more intensive examination of the relationship of PPV to KBSH is warranted before data obtained with KBSH virus are simply assumed for PPV, as has been done recently (Bachman et al, 1979).

Previous studies with PPV have indicated that the production of a visible cytopathic effect with PPV infection of cultured cells was an irregular finding occuring only under "optimum conditions" (Joo and Johnson, 1976), although these optimum conditions were not defined. This study has investigated various parameters of the infection process to describe procedures that routinely result in readily detected cytopathic effect suitable for infectivity assays as well as routine virus production.

Unquestionably, the most significant factor affecting the production of CPE was the degree of confluence of the monolayer at the time of infection. Monolayers with no greater than 50% confluence and very even cell distribution, so as to produce minimal cell to cell contact, produced the most satisfactory results. Both specific and non-specific parvoviral inhibitors have frequently been detected in calf serum (Siegl and Kronaüer, 1980). Removal of such inhibitors by washing monolayers prior to infection was found to aid in the routine production of CPE. As parvoviruses in crude cell lysates have been shown to precipitate in the presence of 25 mM calcium (Tattersall et al, 1976), the use of PBS containing calcium for this washing and for dilution of the viral inoculum produced an added enhancing effect. The concentration of serum in the cell culture medium significantly altered cell growth and thus virus production. Higher serum levels, up to 10% (v/v), produced greater quantities of virus, as well as a faster rate of production. The use of a serum pulse (i.e. sudden shift from low to high serum levels) 24 hours prior to infection seemed to stimulate the highest level of uniform cell division and virus production. For reasons of expediency as well as cost, a 5% serum level was routinely used and proved satisfactory. The addition of lactalbumin hydrolysate was not found to be necessary for successful repeated passage of these cells or to increase virus production. In contrast to the report of Joo and Johnson (1976), maximal viral production was evident at low level virus challenge (approximately 10^{-3} PFU/cell). Such a finding would be expected if the inoculum contained large quantities of defective interfering particles (Muller et al, 1978).

The cytopathological alterations observed in this study at the light microscope level were typical of parvoviral infection as described in detail by Leary and Storz (1980). The mature inclusions observed were almost exclusively of the Cowdry A type. Occasionally inclusions resembling the multifocal type produced by certain strains of bovine parvovirus (Leary and Storz, 1980) could be detected. Coverslip preparations examined at various times PI until the late stage of CPE repeatedly failed to give consistent evidence of significant alterations in cytoplasmic or intranuclear nucleic acid levels as judged by acridine orange staining.This was consistent with the findings of Cartwright et al (1969).

It is peculiar that though parvoviruses such as PPV appear to have icosahedral symmetry, regular lattice aggregation in vivo, as described with other icosahedral viruses such as adenovirus and poliovirus (Joklik, 1980), does not occur. Parvovirus temperature-sensitive mutants have been shown to produce crystals intracellularly (Singer and Rhode, 1978), thereby suggesting a critical surface alteration occuring late in the normal infection cycle, or simply the lack of sufficient numbers of particles. Relatively few infected cells observed electron microscopically demonstrated evidence of progressive viral assembly, although the majority of infected cells contained intranuclear accumulations of dark-staining amorphous material. If, as discussed below, quantities of incomplete particles were present in the inoculum in excess of complete particles, the EM findings would suggest that such incompletes could be infectious with or without the presence of complete particles. These incompletes might code for deficient polypeptides which cannot complete assembly and hence accumulate as amorphous aggregates. Similarly appearing masses of viral protein, as identified by immuno-electron microscopy, are found associated with H-1 parvovirus infected cells in increasing quantities during the later stages of infection (Singer and Rhode, 1978).

Information concerning the purification of PPV is extremely limited. Only two reports have been published describing attempts to purify PPV (Mayr et al, 1968; Morimoto et al, 1972b) since its original discovery in 1966 (Mayr and Mahnel). Both methods relied solely on isopycnic banding in CsCl gradients to achieve purification and neither method assessed purity other than by electron microscopic observation. Various modifications of the wide range of procedures reported for other parvoviruses were evaluated through the course of this study to discover a technique that would produce a maximal band of virus in buoyant density gradients which would contain only the three typical parvovirus polypeptides as determined by SDS-PAGE analysis. Two procedures were adopted for routine use, one producing maximum quantity and the other producing maximum quality. The procedure described by Hallauer et al (1971) utilizing alkaline glycine buffer and freon extraction of infected cells was slightly modified by the addition of a sonication step to disrupt infected cells and a CsCl-sucrose step gradient for the final step. Although maximal quantities of virus were recovery by this approach, as judged by electron microscopy, SDS-PAGE analysis indicated the presence of atypical polypeptides in quantities in excess of the apparent structural polypeptides. The modified Hallauer procedure would seem most suitable for recovery of virion DNA where further degradation of proteins was anticipated. In this study, the most highly purified virus was obtained by use of the procedure described by Tattersall et al (1976), or the modification described by Bourgignon et al (1976) incorporating trypsin treatment of the cell lysate. The analysis of virus suspensions prepared in this study by the shortened Tattersall procedure indicated the presence of residual contaminating cellular DNA. The two final purification steps, namely treatment with micrococcal nuclease and subsequent sucrose sedimentation, would appear mandatory to obtain maximum purity. The greatest drawback of this purification scheme apart from its complexity, is the poor final yield of approximately 10% as reported by Tattersall et al and confirmed in this present study (Table 4). The reasons for this poor yield were obvious from examination of the the procedure itself. The extremely mild method of initial cellular disruption effectively served to leave membranes and organelles intact, but was quite ineffective compared to sonication or detergent treatment in releasing intracellular virus. The subsequent pelleting of the virus had a drastic effect on virion stability apparently affecting empty, immature and incompletely filled particles to the greatest extent. When such weakened particles are subjected to the massive gravitational force and high osmotic pressure used in CsCl isopycnic centrifugation, it must be expected that considerable loss of infectivity can occur.

Following centrifugation of the parvovirus MVM in an unique CsCl-sucrose step gradient, Tattersall et al described the presence of only two discrete bands - an upper band containing empty capsids and a lower band containing full particles. The lower band found in the present study would seem to correspond to Tattersall et al's two bands. The presence of predominantly incomplete particles in the PPV preparations, with sedimentation rates and buoyant densities between those of complete and empty virions, could account for the lack of resolution of this lower band into two distinct bands. The intermediate band, as judged by electron microscopy, HA titration and SDS-PAGE, consisted of a relatively small quantity of virus firmly adhered to cellular proteins, resulting in a slower sedimentation rate and lower buoyant density and a higher position in the gradient than pure virus. These lighter buoyant density particles most likely were in the process of assembly at the time of virus harvest and were complexed with cellular nucleoprotein and the enzymes involved in virion DNA packaging. As Tattersall et al described only the direct collection of the visible bands from CsCl-sucrose step gradients, and not a fractional assay for HA activity, the significance of the upper band of soluble material was ignored in their study.

The statement by Tattersall et al (1976) that the "virus was sedimented to equilibrium" after ultracentrifugation in the CsCl-sucrose gradient must be challenged. Assessment of the buoyant density profile of such gradients by optical refractometric methods in fact indicate that a static buoyant density profile was not achieved. As multiple attempts to reproduce visible viral bands from the initial gradients in isopycnic CsCl, consistently failed, it would seem that an aggregation phenomenon occurred during passage of virions through the sucrose layer and was involved with the formation of this visible band.

Adequate methods of assessing the infectivity levels of PPV suspensions have not previously been described. Virtually all optimized parvoviral production systems make use of the plaque assay for infectivity titration due to its ease of reproducibility and distinct endpoint (Tattersall, 1972; Siegl and Kronauer, 1980). Attempts to interpret the results of experimental infection studies without adequate objective evaluation of infectivity levels, as have been reported previously (Johnson and Collings, 1969; Johnson and Collings 1971; Cartwright et al 1971; Hogg et al, 1977) are obviously quite futile and the wide variation in results reported are certainly to be expected. Furthermore, none of the attempted experimental infections made use of purified virus and no attempt was made to assess the proportion of non-infectious antigen (i.e. soluble virion components and incomplete particles) present in the inoculum. The extremely subjective nature of the inclusion body, fluorescent focus or poorly designed TCID50 assays make infectivity titration by such procedures very unreliable. The extreme dependence of parvoviruses on cellular functions expressed only transiently during the cell cycle makes quantitation of infected cells in fluid medium highly unreliable unless strict conditions of cell synchrony and processing times are used. Such conditions are unsatisfactory for routine use. The wide variation in the ratio of HA:infectivity noted between extracellular tissue culture fluid and lysates of infected cells clearly indicates that estimation of infectivity by simple HA titration is unjustified.

Examination of the growth curve of PPV in the ST cell line (Figure 10) revealed that the period of maximum increase in infectious virus ends at 36 h PI, long before CPE was visible (72 hours PI). 36 h PI would, then, be the ideal time to harvest virus for maximum levels of complete virions. During the period 36-48 hours PI, viral hemagglutinin continued to be produced at a logarithmically increasing rate, while the rate of infectivity production tapered off. This observation indicated an accumulation of soluble viral components which were not being effectively incorporated into virions and/or the production of defective virions that were incapable of causing productive infection. As cell cultures were routinely harvested 3-5 d PI when CPE was evident, preparations in this study contained considerable quantities of such products. Attempts to harvest cultures at earlier times PI resulted in an unacceptable reduction in the total virus recovered. In the study performed by Tattersall et al (1976), cultures were routinely harvested at the earlier stage and the resulting preparations contained almost exclusively complete and empty virions. Tattersall et al (1976) effectively circumvented the problem of low yield by routinely radiolabelling virus and by using spinner cultures of suitable cells which allowed large quantities of cells to be easily managed. It would appear that the soluble components and incomplete particles accumulate late in the infectious cycle when cells are quite acidotic and toxic.

Hemagglutinating activity is an invariable property of all parvoviruses (Bachman et al, 1979). Hemagglutination by porcine parvovirus is a simple means to differentiate PPV from enteroviruses which are only slightly larger but less consistently hemagglutinate (Douglas, 1977). It is of interest to note that no parvovirus described to date agglutinates erythrocytes of the homologous host species. Such a property would prevent hematogenous spread of virus as adherence to enucleated erythrocytes, which are by far the predominant cell type in peripheral blood, could only result in aborted infection. The presence of receptors for parvoviral hemagglutinin on erythrocytes from a particular species would seem to be mutually exclusive with the presence of receptors on nucleated cells of that same species which allow viral penetration and subsequent infection. PPV has been shown to strongly agglutinate human erythrocytes (Cartwright et al, 1969) and thus would not be expected to infect human cell lines as Hallauer et al (1971) have suggested.

The use of immunoelectrophoretic techniques to identify the location of parvoviral antigen in gradients has been used infrequently. Presumably this is due to the relatively large quantity of protein required for such procedures compared to the use of radiolabelling or simple HA screening. Bloom et al (1980) have recently reported the successful use of CIE to identify parvoviral antigen in fractions collected from CsCl isopycnic gradients containing virus purified by the Tattersall procedure, in a very similar manner to the rocket immunoelectophoresis analysis used in this study. Bloom et al (1980) also found an isolated peak, which similarly coincided with maximum HA activity, occurring in the high buoyant density range of 1.42-1.44 gm/mL, suggesting that heavy full virions are the least stable. However, Bloom et al (1980) also reported antigen containing fractions at the lowest buoyant density ranges of their gradient. As Bloom et al (1980) ran their gradients for a longer period, this may have resulted from the movement of lysed capsids to the buoyant density of isolated protein (i.e. 1.28 gm/mL) or the breakdown of slightly more stable incomplete virions found at this lower buoyant density. As CIE, unlike rocket immunoelectrophoresis, is not quantitative it could not be determined from the report by Bloom et al (1980) if maximum quantities of antigen were detected at the high buoyant density peak as in the present study, or at the low buoyant density region.

Two-dimensional immunoelectrophoresis (2D) is a widely applicable technique to identify and analyze mixtures of antigens (Axelsen et al, 1973). An attempt was made in this study to analyze the band of material found at the top of CsCl-sucrose step gradients following centrifugation. The fact that this material showed relatively high HA titres, despite the lack of any significant number of virions as judged by electron microscopy, had a buoyant density and/or sedimentation rate suggestive of protein or lipoprotein, and gave positive CIE tests suggested that it might be composed largely of PPV soluble components. The precipitation profile produced by 2D analysis suggests that the fractions contained two antigens slightly differing in electrophoretic mobility but sharing an antigenic determinant. The fuzzy nature of the profile and the aggregation at the edge of the well would suggest residual lipids present in the antigen pool (L. Tsang, personal communication). As the electron micrographs would indicate that this material originates from the amorphous band lining the internal edge of the nuclear membrane of infected cells, tightly adherent membranous lipids would be expected in such pools. Repeated freon treatment apparently removed the complete antigen-lipid complex instead of simply extracting the lipid as was hoped. It is possible that clearer profiles could be obtained with this antigen pool following a more intensive investigation of suitable detergent treatment to emulsify the lipids. Only one attempt was made to use this approach to enhance results, but prolonged treatment apparently destroyed the antigenicity of the proteins.

Limited use has been made of ultraviolet absorption spectra to analyze the purity of parvoviral preparations. The application of this procedure relies upon the premise that nucleic acid preferentially absorbs light at 260 nm wavelength and protein at 280 nm. Thus any homogeneous purified virus species should demonstrate a characteristic E260/E280 ratio, presuming that there is a constant ratio of nucleic acid to protein. The presence of defective particles, containing less than a complete genome, would obviously impair such an analysis. The only E260/E280 ratio published for a parvovirus is 1.38 for MVM (Tattersall et al, 1976). Whether the difference between that value and the one determined in this study (1.45) is due to species variation or minor contamination of the PPV preparation by residual nucleic acid is speculative. However, as residual nucleic acids were removed from the MVM preparation by enzyme treatment and sucrose sedimentation prior to UV spectrum analysis, the latter explanation appears more likely.

Sucrose sedimentation has been reported to be an effective means of separating empty and full virions, which demonstrate widely differing sedimentation rates (Tattersall et al, 1976; Richards et al, 1977), as well as complete from incomplete particles, which show more similar sedimentation rates but exhibit two discrete peaks (Muller et al, 1978). The results of the sucrose sedimentation performed in the present study were very similar to the sedimentation profile produced by Muller et al and would suggest the presence of a broad range of incomplete particles.

Alkaline sucrose sedimentaion proved to be the preferable method for isolation of virion DNA, as has been found in various other studies (Bourgignon et al,1976; Faust and Ward, 1979). The sedimentation profile obtained for PPV DNA showed a single major peak, as described in the other studies. However, the peak observed was considerably broader, despite the use of the same conditions of sedimentation. Once again, this would suggest the presence of particles with less than a full complement of DNA. This main peak was preceded by a minor peak presumed to be residual cellular DNA. The minor peak was not observed in the earlier parvoviral studies (Bourgignon et al, 1976) which employed digestion of this material with micrococcal nuclease prior to virion lysis.

The only purification technique producing virus containing only the three characteristic parvoviral polypeptides (Tattersall, 1978a) was that of Tattersall et al (1976), despite the fact that various techniques produced virus showing a high degree of purity on electron microscopic examination. The SDS-PAGE technique readily differentiated between the B and C polypeptides even though the polypeptides differ in apparent molecular weight by only 5%. Although trypsin treatment causes conversion of the B polypeptide to a polypeptide that co-migrates with the C polypeptide, as was shown in the present study by considerable increase in the quantity of C present when trypsin was used during purification, it has yet to be determined if this occurs in vivo. The presence in less effectively purified preparations of a polypeptide of molecular weight 55,000 corresponds to results found with several other parvoviruses (Tattersall, 1978). The presence of this polypeptide has been shown to vary with the host cell line (Tattersall et al, 1976). In the present study such a polypeptide was found in virus propagated in PPK cells purified by freon extraction and CsCl equilibrium centrifugation, but not to any significant extent in virus grown in ST cells and purified by the Tattersall procedure. Similarly, a polypeptide that would appear to correspond to the 72,500 dalton species in the present study has also been resolved on PAGE when a less efficient purification than the Tattersall procedure is used (Bates et al, 1978). This polypeptide is thought to be a cellular DNA polymerase

(Tattersall, 1978a).

Numerous techniques have been employed to verify the single-stranded nature of parvoviral nucleic acid such as invariance of its sedimentation characteristics in both neutral and alkaline sucrose as well as cleavage by single-strand specific nucleases (Berns and Hauswirth, 1978). In the present study the single-stranded nature was confirmed by three techniques. Electron microscopic comparison of PPV DNA to a readily distinguishable circular double-stranded phage DNA, although a somewhat variable and subjective evaluation, indicated a single-stranded appearance. A more objective assessment was obtained from the results of the thermal denaturation experiment which confirmed the expected single-stranded configuration. The most conclusive evidence, however, was the distinct differential staining properties of isolated PPV DNA on agarose gel electrophoresis, when compared to a double-stranded control. Furthermore, the consistent appearance of PPV nucleic acid on such gels both before and after attempted degradation by ribonuclease, confirmed that PPV contains a DNA genome. Unfortunately, due to a lack of more appropriate controls, an exact evaluation of the size of the complete PPV genome was not possible. The results of both the electron microscopic study and agar gel electrophoresis would suggest that this figure lies in the range of 1.05-1.7 million daltons, which overlaps the currently recognized range for the genus Parvovirus 1.5-2.2

million daltons (Bachman et al, 1979).

The great range of DNA lengths observed by electron microscopy makes the characteristics of populations of PPV virions isolated throughout this study easily explainable. Virtually all parvoviral DNA preparations exhibit this heterogeneous nature when assessed electron microscopically (Singer and Rhode, 1977a; Shahrabadi et al, 1977) although analysis of these same preparations by gel electrophoresis or sucrose sedimentation deceptively suggests a more homogeneous nature (Faust and Ward, 1979). Parvoviral culture systems optimized for production of complete particles on occassion demonstrate as many as 30% incomplete genomes (Bourgignon et al, 1976). However, levels of defective particles as high as were observed in the present study (94%) have not been previously reported. The reason for such high levels of incomplete particles would seem most likely to be associated with the practice of multiple undiluted passage used earlier in the study to produce the stock virus inoculum, harvesting virus at a late stage in the infectious cycle and the nature of the host cell system (Muller et al, 1978).

The structural nature of parvoviruses has yet to be clearly defined, although it is believed that they belong to the T=3 class of viruses and probably possess a total of 32 capsomers comprised of 20 hexons and 12 pentons (Joklik, 1980). The exact distribution of the known structural polypeptides within the virion similarly remains to be clearly elucidated. Salzman and Koczot (1978) have isolated nucleoprotein cores of the parvovirus KRV by alkaline sucrose sedimentation at pH 10 which demonstrated only the high molecular weight or A polypeptide. Tattersall (1978a) has proposed that as considerable evidence has accumulated to suggest that the B polypeptide is converted post-translationally to the C polypeptide following DNA packaging, these two polypeptides are presumably found on the surface of the virion. Although little information concerning capsomer structure was apparent from the present study, numerous micrographs indicated dissociation of particles into inner core structures and outer shells. These inner cores would be expected to consist of the DNA genome surrounded by a thin protein layer and thus have a higher buoyant density than complete virions. Cores of a similar size and exhibiting a high buoyant density in CsCl have previously been described in PPV preparations without any explanation (Mayr et al, 1967). As a detailed theory of parvovirus assembly has yet to be proposed, it is not certain whether these core structures have any relevance in vivo or occur simply as artifacts following harsh treatment of unstable virions.

The results of the serological survey for antibodies to PPV demonstrate that PPV infection is very common in central Alberta. The HI tests did not consistently produce distinct endpoints and variations in titres were noticed when tests were repeated, although results were consistently either positive or negative. The test as described by Joo et al (1976b) is tedious, time consuming and prone to dilutional errors when large numbers of sera must be processed by hand. The excellent correlation of the HI and CIE results, suggest that CIE could be readily applied to provide quick, clearcut evidence of previous PPV infection in clinical diagnostic laboratories. The simplicity of the procedure, combined with the minimal requirement for glassware, reagents and technician's time would make it relatively inexpensive and applicable to widescale or frequent testing, such as is necessary at artificial insemination stations and in gnotobiotic herds.

On the basis of information accumulated in this study, it can be concluded that PPV possesses a DNA and protein content consistent with its classification in the genus Parvovirus. The swine testicle cell line used in this study for the propagation of PPV demonstrates properties adequate for uncomplicated production of utilizable quantities of this virus for further characterization studies, the production of diagnostic antigens and possibly commercial vaccines as well.

It has been shown in this study that PPV can be purified and quantitated using procedures that are rapidly becoming standard in parvoviral study. The application of the methods and results in this study will allow more reliable data to be obtained concerning the nature and control of porcine parvoviral infection.

BIBLIOGRAPHY

Abianti, F. and Warfield, M. 1961. Recovery of a hemadsorbing virus (HADEN) from the gastrointestinal tract of calves. Virology 14: 288-293.

Anderson, G. and Anderson, N. 1968. Density at 25 C. of cscl solution as a function of refractive index. *In:* Handbook of Biochemistry, pp.252-256, edited by Sober, H., The Chemical Rubber Co., Cleveland, Ohio

Axelsen, N., Kroll, J. and Weeks, B. 1973. A manual of quantitative immmunoelectrophoresis. Methods and applications. Scand. J. Immun. I: Supp. I

Bachman, P. 1970. Parvoviren beim schwein. Zentbl. VetMed. 17B: 192-194.

Bachman, P. 1972. Porcine parvovirus infection in vitro: A study model for the replication of parvoviruses. I Replication at different temperatures. Proc. Soc. Exp. Biol. Med. 140: 1369-1374.

Bachman, P., Hoggan, M., Melnick, J., Pereira, H. and Vago, C. 1975. Parvoviridae. Intervirology 5: 83-92.

Bachman, P., Hoggan, M., Kurstak, E., Melnick, J., Pereira, H., Tattersall, P. and Vago, C. 1979. Parvoviridae: second report. Intervirology 11: 248-254.

Bachman, P., Sheffy, B. and Vaughn, J. 1975b. Experimental in utero infection in fetal pigs with a porcine parvovirus. Infect. Immun. 12: 455-460.

Bates, R., Kuchenbuch, C., Patton, J. and Stout, E. 1978. DNA-polymerase activity in parvovirus-infected cells. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 367-382, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Berns, K. and Hauswirth, W. 1978. Parvovirus DNA structure and replication. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 13-32, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Binn, L., Lazar, E., Eddy, G. and Kajima, M. 1968. Minute viruses of canines. Abstr. 68th Annu. Meet. Am. Soc. Microbiol. 98: 161- 166.

Binn, L., Lazar, E., Eddy, G. and Kajima, M. 1970. Recovery and characterization of a minute virus of canine. Infect. Immun. 1: 503-508.

Bloom, M. Race, R. and Wolfinbarger, J. 1980. Characterization of aleutian disease virus as a parvovirus. J. Virol. 35: 836-843.

Boggs, J., Melnick, J., Conrad, M. and Felsher, B. 1970. Viral hepatitis clinical and tissue culture studies. J. Am. Med. Assoc. 214: 1041-1047.

Bourgignon, G., Tattersall, P. and Ward, D. 1976. DNA of minute virus of mice: Self-priming, nonpermuted, single-stranded genome with a 5′-terminal hairpin duplex. J. Virol. 20: 290-306.

Bourne, F., Curtis, J., Johnson, R. and Collings, D. 1974.

Antibody formation in porcine fetuses. Res. Vet. Sci. 16: 223-227.

Carter, B., 1978. Parvovirus transcription. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 33-52, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Cartwright, S. and Huck, R. 1967. Viruses isolated in association with herd infertility, abortions and stillbirths in pigs. Vet. Rec. 81: 196-197.

Cartwright, S., Lucas, M. and Huck, R. 1969. A small haemagglutinating porcine DNA virus. I. Isolation and properties. J. Comp. Path. 79: 371-377.

Cartwright, S., Lucas, M. and Huck, R. 1971. A small haemagglutinating porcine DNA virus. II. Biological and serological studies. J. Comp. Path. 81: 145-155.

Chow, M. and Ward, D. 1978. Comparison of the terminal nucleotide structure of the DNA of non-defective parvoviruses. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 205-218, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Clinton, G. and Hayashi, M. 1975. The parvovirus mvm: Particles with altered structural proteins. Virology 66: 261-267.

Clinton, G. and Hayashi, M. 1976. The parvovirus mvm: A comparison of heavy and light particle infectivity and their conversion in vitro. Virology 74: 57-63.

Coakley, W. and Smith, V. 1972. Porcine parvovirus in western Australia. Aust. Vet. J. 48: 536-541

Crawford, L. 1966. A minute virus of mice. Virology 13: 515-522.

Croghan, D., Matchett, A. and Koski, T. 1973. Isolation of porcine parvovirus from commercial trypsin. Appl. Microbiol. 26: 431-433.

Cutlip, R. and Mengeling, W. 1975a. Experimentally induced infection of neonatal swine with porcine parvovirus. Am. J. Vet. Res. 36: 1179-1182.

Cutlip, R. and Mengeling, W. 1975b. Pathogenesis of in utero infection: Experimental infection of eight and ten week old porcine fetuses with porcine parvovirus. Am. J. Vet. Res. 36: 1751-1754.

Dalldorf, G. 1960. Viruses and human cancer. Bull. N.Y. Acad. Med. 36: 795-809.

Darbyshire, J. and Roberts, D. 1968. Some respiratory virus and mycoplasma infections of animals. J. Clin. Path. 21, Suppl. 2: 61-87.

Davis, R., Simon, M. and Davidson, N. 1971. Electron microscopic hetero-duplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. 21: 413-428.

De la Maza, L. and Carter, B. 1980. Heavy and light particles of adeno-associated virus. J. Virol. 33: 1129-1137.

De la Maza, L. and Carter, B. 1978. DNA structure of incomplete adeno-associated virus particles. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 193-204, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Derzsy, D. 1967. A viral disease of goslings. I. Epidemiological, clinical, pathological and aetiological studies. Acta. Vet. Hung. 17: 443-450.

Dolin, R., Blacklow, N., Dupont, H., Buscho, R., Wyatt, R., Kasel, J., Hornick, R. and Chanock, R. 1972. Biological properties of the Norwalk agent of acute nonbacterial gastroenteritis. Proc. Soc. Exp. Biol. Med. 140: 578-584.

Donaldson-Wood, C. Joo, H. and Johnson, R. 1977. The effect on reproductive performance of porcine parvovirus infection of a susceptible herd. Vet. Rec. 100: 237-239.

Douglas, R. 1977. Comparative diagnosis of picornavirus (enterovirus and rhinovirus) infections. *In:* Comparative Diagnosis of Viral Diseases I, edited by Kurstak, E. and Kurstak, C. pp. 343-383. Academic Press, New York.

Dulac, G., Ruckerbauer, G. and Boulanger, P. 1977. Transmissible gastro-enteritis: Demonstration of the virus from field specimens by means of cell culture and pig innoculation. Can. J. Comp. Med. 41: 357-363.

EL Dadah, A., Smith, K., Squire, R., Santos, G. and Melby, E. 1967. Viral hemorrhagic encephalopathy of rats. Science 156: 392-402.

Faust, E. and Ward, D. 1979. Incomplete genomes of the parvovirus minute virus of mice: Selective conservation of genome termini, including the origin for DNA replication. J. Virol. 32: 276-292.

Gomori, G. 1955. *In:* Methods in Enzymology, Vol. I, edited by Colowick, S. and Kaplan, N., pp. 138-146. Acad. Press, New York.

Green, M. and Pina, M. 1964. Biochemical studies of adenovirus multiplication. VI. Properties of highly purified tumorigenic human adenoviruses and their DNA's. Proc. Nat. Acad. Sci. 51: 1251-1259.

Hallauer, C. and Kronauer, G. 1965. Extraction of cell-associated virus without damage of the culture. Arch. Ges. Virusforsch. 15: 434-440.

Hallauer, C., Kronauer, G. and Siegl, G. 1971. Parvoviruses as contaminants of human cell lines. I. Virus isolations from 1960-1970. Arch. Ges. Virusforsch. 35: 80-90.

Hammon, W. and Enders, J. 1939. A virus disease of cats principally characterized by aleukocytosis, enteric lesions and the presence of intranuclear inclusion bodies. J. Exp. Med. 69: 327-352.

Hogg, G. Lenghaus, C. and Forman, A. 1977. Experimental porcine parvovirus infection of foetal pigs resulting in abortion, histological lesions and antibody formation. J. Comp. Path. 87: 539-549.

Hoggan, M., Sears, J., Thomas, G. and Roy, A. 1978. Biological properties of rat virus variants. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 119-130, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Huygelen, C. and Peetermans, J. 1967. Isolation of a haemagglutinating picornavirus from a primary swine kidney cell culture. (Brief report). Arch. Ges. Virusforsch. 20: 260-262.

Hsiung, G. 1973. Hematoxylin-eosin staining. *In:* Diagnostic Virology, pp. 41-43. Yale University Press Ltd., London.

Johnson, R. 1965. Feline panleukopaenia. I. Identification of a virus associated with the syndrome. Res. Vet. Sci. 6: 466-473.

Johnson, R. 1969. A search for parvoviridae (picodnaviridae). Vet. Rec. 84: 19-20.

Johnson, R., 1973. Isolation of swine parvovirus in

Queensland. Aust. Vet. J. 49: 157-159.

Johnson, F. Blacklow, N. and Hoggan, M. 1972. Immunological reactivity of antisera prepared against the sodium dodecyl sulfate treated structural polypeptides of adeno-associated virus. J. Virol. 9: 1017-1026.

Johnson, R. and Collings, D. 1969. Experimental infection of piglets and pregnant guilts with a parvovirus. Vet. Rec. 85: 446-447.

Johnson, R. and Collings, D. 1971. Transplacental infection of piglets with a porcine parvovirus. Res. Vet. Sci. 12: 570-572.

Johnson, R., Donaldson-Wood, C., Joo, H. and Alexander, U. 1976. Observations on the epidemiology of porcine parvovirus. Aust. Vet. J. 52: 80-84.

Joklik, W. 1980. The virus multiplication cycle. *In:* Principles of Animal Virology, pp. 76-120 Appleton-Century-Crofts, New York.

Joo, H., Donaldson-Wood, C. and Johnson, R. 1976a. Rapid diagnostic techniques for detection of porcine parvovirus infection in mummified foetuses. Aust. Vet. J. 52: 51-52.

Joo, H., Donaldson-Wood, C. and Johnson, R. 1976b. A standardised haemagglutination inhibition test for porcine parvovirus antibody. Aust. Vet. J. 52: 422-424.

Joo, H., Donaldson-Wood, C. and Johnson, R. 1976c. Observations on the pathogenesis of porcine parvovirus infection. Arch. Virol. 51: 123-129.

Joo, H., Donaldson-Wood, C. and Johnson, R. 1976d. Antibody to porcine, feline and rat parvovirus in various animal species. Res. Vet. Sci. 21: 112-113.

Joo, H. and Johnson, R. 1976. Porcine parvovirus: A review. Vet. Bulletin 46: 653-660.

Joo, H. and Johnson, R. 1977. Serological responses in pigs vaccinated with inactivated porcine parvovirus. Aust.

Vet. J. 53: 550-552.

Kasza, L., Shaddock, J. and Christofinis, G. 1972. Establishment, viral susceptibility and biological characteristics of a swine kidney cell line sk-6. Res. Vet. Sci. 13: 46-51.

Kilham, L. and Oliver, 1959. A latent virus of rats isolated in tissue culture. Virology 7: 428-436.

Kongsvik, J., Hopkins, M. and Ellem, K. 1979. Subfractionation of cscl-purified h-1 parvovirus on metrizamide gradients. Virology 96: 646-651.

Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lavelle, G. and Mitra, S. 1978. Double-helical regions in Kilham rat virus DNA. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp.219-230, Cold Spring Harbour Press, Cold Spring

Harbour, N.Y.

Leary, J. and Storz, J. 1980. Nucleopathic changes in parvovirus-infected, cultured cells. Exp. Mol. Path. 32: 188-200.

Linser, P. and Armentout, R. 1978. Binding of minute virus of mice to cells in culture. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 151-160, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Lum, G. and Schreiner, A. 1963. Study of a virus isolated from a chloroleukemic Wistar rat. Cancer Res. 23: 1742-1753.

Lwoff, A. and Tournier, P. 1966. The classification of viruses. A. Rev. Microbiol. 20: 45-74.

Marusyk, R. and Cummings, G. 1978. Comparative polypeptide patterns of representative human and nonhuman adenoviruses. Intervirology 10: 356-360.

Marusyk, R. and Sergeant, A. 1980. A simple method for dialysis of small-volume samples. Anal. Biochem. 105: 403-404.

Marviv, P. and Hohn, A. 1969. Filamentous bacterial viruses. Bact. Rev. 33: 172-209.

Matsunaga, Y., Matsuno, S. and Mukoyama, J. 1977. Isolation and characterization of a parvovirus of rabbits. Infect. Immun. 18: 495-501.

May, P. and May, E. 1970. The DNA of kilham rat virus. J. Gen. Virol. 6: 437-439.

Mayor, H. and Melnick, J. 1966. Small deoxyribonucleic-acid-containing viruses (picodnavirus group). Nature 210: 331-332.

Mayr, A. and Mahnel, H. 1966. Weitere untersuchungen uber die zuchtung von schweinespestvirus in zelkulturen mit cytopathogenem effekt. Zentr. Bakteriol. Abt. I. Orig. pp. 199-399.

Mayr, A., Bachman, P., Sheffy, B. and Siegl, G. 1967. Electron optical and buoyant density studies of hog cholera virus. Arch. Ges. Virusforsch. 21: 113-119.

Mayr, A., Bachman, P., Siegl, G., Mahnel, H. and Sheffy, B. 1968. Characterization of a small porcine DNA virus. Arch. Ges. Virusforsch. 25: 38-51.

McMaster, G. and Carmichael, G. 1977. Analysis of single and double stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Nat. Acad. Sci. 74: 4835-4838.

Mengeling, W. 1972. Porcine parvovirus: Properties and prevalence of a strain isolated in the United States. Am. J. Vet. Res. 33: 2239-2248.

Mengeling, W. 1975a. Porcine parvovirus: Frequency of naturally occurring transplacental infection and viral contamination of fetal porcine kidney cell cultures. Am. J. Vet. Res. 36: 41-44.

Mengeling, W. 1975b. Porcine parvovirus. *In:* Diseases of Swine, Fourth Edition. pp. 432-446, Edited by Dunne, H. and Leman, A., Iowa State Press, Ames, Iowa.

Mengeling, W. 1979. Prenatal infection following maternal exposure to porcine parvovirus on either the seventh or fourteenth day of gestation. Can. J. Comp. Med. 43: 106-109.

Mengeling, W., Brown, T., Paul, P. and Gutekunst, D. 1979. Efficacy of inactivated virus vaccine for prevention of porcine parvovirus induced reproductive failure. Am J. Vet. Res. 40: 204-207.

Mengeling, W. and Cutlip, R. 1975. Pathogenesis of in utero infection: Experimental infection of five-week-old porcine fetuses with porcine parvovirus. Am. J. Vet. Res. 36: 1173-1177.

Mengeling, W. and Cutlip, R. 1976. Reproductive disease experimentally induced by exposing pregnant guilts to porcine parvovirus. Am. J. Vet. Res. 37: 1393-1400.

Mengeling, W., Cutlip, R., Wilson, R., Parks, J. and Marshall, R. 1975. Fetal mummification associated with porcine parvovirus infection. J. Am. Vet. Med. Ass. 166: 993-995.

Morimoto, T., Kurogi, H., Miura, Y., Sugimori, T. and Fujisaki, Y. 1972a. Isolation of Japanese encephalitis virus and a haemagglutinating DNA virus from the brain of stillborn piglets. Natn. Inst. Anim. Hlth. Qt., Tokyo 12: 127-136.

Morimoto, T., Fujisaki, Y., Ito, Y. and Tanaka, Y. 1972b. Biological and physiochemical properties of porcine parvovirus recovered from stillborn piglets. Natn. Inst. Anim. Hlth. Qt., Tokyo 12: 137-144.

Mulder, C., Sharp, P., Delius, H. and Petterssen, U. 1974. Specific fragmentation of DNA of adenovirus serotypes 3,5,7 and 12 and adeno-simian virus 40 hybrid ad2+nd1 by restriction endonuclease R-Eco R1. J. Virol. 14: 68-77.

Muller, H., Gautschi, M. and Siegl, G. 1978. Defective particles of parvovirus lu III. *In:* Replication of

Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 231-240, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Norrby, E. 1966. The relationship between the soluble antigens and the virion of adenovirus type 3. II. Identification and characterization of an incomplete hemagglutinin. Virology 32: 489-502.

Patton, J., Stoutt, E. and Bates, R. 1979. Transcription of the bovine parvovirus genome in isolated nuclei. J. Virol. 30: 917-922.

Paul, P., Mengeling, W. and Brown, T. 1979. Replication of porcine parvovirus in peripheral blood lymphocytes, monocytes and peritoneal macrophages. Infect. Immun. 25: 1003-1007.

Payne, F., Shellabarger, C. and Scmidt, R. 1963. A virus from mammary tissue of a rat treated with X-ray or methylcholanthrene (MC). Proc. Am. Assoc. Cancer Res. 4: 51-62.

Peterson, J., Dale, R., Karess, R., Leonard, D. and Ward, D. 1978. Comparison of parvovirus structural proteins: Evidence for post-translational modification. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 431-445, Cold Spring Harbour Press, Cold Spring Harbour, N.Y..

Pini, A. 1975. Porcine parvovirus in southern Africa. J. S. Afri. Vet. Ass. 46: 241-244.

Pirtle, E. 1974. Titration of two porcine respiratory viruses in mammalian cell culture by direct fluorescent antibody staining. Am. J. Vet. Res. 35: 249-250.

Porter, D. and Larsen, A. 1974. Aleutian disease virus of mink. Prog. Med. Virol. 18: 32-47.

Redman, D., Bohl, E. and Ferguson, L. 1974. Natural and experimental infections of the porcine fetus and prevalence in mature swine. Infect. Immun. 10: 718-723.

Reed, L. and Muench, H. 1938. A simple method of estimating fifty per cent endpoints. Amer. J. Hyg. 27: 493-497.

Reynolds, E. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

Rhode, S. 1973. Replication process of the parvovirus h-1. I. Kinetics in a parasynchronous cell system. J. Virol. 11: 856-861.

Rhode, S. 1977. Replication process of the parvovirus h-1: VI Characterization of a replication terminus of h-1 replicative form DNA. J. Virol. 21:694-712.

Rhode, S. 1978. Defective interfering particles of parvovirus h-1. J. Virol. 27: 347-356.

Richards, R., Linser, P. and Armentout, R. 1977. Kinetics of assembly of a parvovirus, minute virus of mice, in synchronized rat brain cells. J. Virol. 22: 778-793.

Rodeffer, H., LeMan, A., Dunne, H., Cropper, M. and Sprecher, D. 1975. Reproductive failure in swine associated with maternal seroconversion for porcine parvovirus. J. Am. Vet. Med. Ass. 166: 991-992.

Rose, J. 1974. Parvovirus reproduction. In: Comprehensive Virology 3, Reproduction, DNA Animal Viruses, edited by Fraenkel-Conrat, H. and Wagner, R. pp. 1-61, Plenum Press, New York.

Rovozzo, G. and Burke, C. 1973. Tissue culture preparation. *In:* A Manual of Basic Virological Techniques. pp. 38-63. Prentice-Hall, Inglewood Cliffs, N.J.

Ruckerbauer, G., Dulac, G., and Boulanger, R. 1978. Demonstration of parvovirus in Canadian swine and antigenic relationships with isolates from other countries. Can. J. Comp. Med. 42: 278-285.

Salzman, L. and Jori, L. 1970. Characterization of the Kilham rat virus. J. Virol. 5: 114-121.

Salzman, L. and Koczot, F. 1978. Isolation of nucleoprotein from the parvovirus krv. Virology 84: 434-442.

Saif, L., Rotavirus-like, calicivirus-like and 23-nm. virus-like particles associated with diarrhea in young pigs. J. Clin. Micro. 12: 105-111.

Shahrabadi, M., Cho, H. and Marusyk, R. 1977. Characterization of the protein and nucleic acid of aleutian disease virus. J. Virol. 23: 353-362.

Siegl, G. 1972. Parvoviruses as contaminants of permanent human cell lines. V. The nucleic acid of kbsh-virus. Arch. Ges. Virusforsch. 37: 267-274.

Siegl, G. and Gautschi, M. 1973a. The multiplication of parvovirus lu III in a synchronized culture system. I. Optimal conditions for virus replication. Arch. Ges. Virusforsch. 40: 105-118.

Siegl, G. and Gautschi, M. 1973b. The multiplication of parvovirus lu III in a synchronized culture system. II.

Biochemical characteristics of virus replication. Arch. Ges. Virusforsch. 40: 119-127.

Siegl, G. and Kronauer, G. 1980. A plaque assay for feline panleukopenia virus. J. Gen. Virol. 46: 211-218.

Siegl, G., Hallauer, C. and Novak. 1972. Parvoviruses as contaminants of permanent human cell lines. IV. Multiplication of kbsh-virus in kb-cells. Arch. Ges. Virusforsch. 36: 351-362.

Siegl, G., Hallauer, C., Novak, A. and Kronauer, G. 1971. Parvoviruses as contaminants of permanent human cell lines. II. Physical properties of the isolated viruses. Arch. Ges. Virusforsch. 35: 91-103.

Singer, I. and Rhode, S. 1977a. Replication process of the parvovirus h-1. VII. Electron microscopy of replicative-form DNA synthesis. J. Virol. 21: 713-723.

Singer, I. and Rhode, S. 1977b. Ultrastructural studies of H1 parvovirus replication. IV. Crystal development and structure with the temperature sensitive mutant ts-1. J. Virol. 24: 343-352.

Singer, I. and Rhode, S. 1977c. Ultrastructural studies of H1 parvovirus replication. V. Immunocytochemical demonstration of separate chromatin-associated and inclusion-associated antigens. J. Virol. 24: 353-362.

Singer, I and Rhode, S. 1978. Electron microscopy and cytochemistry of H-1 parvovirus intracellular morphogenesis. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 479-504 Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Sinsheimer, R. 1959. A single stranded deoxyribonucleic acid from bacteriophage ØX174. J. Mol. Biol. 1: 43-53.

Sulochana, S. and Derbyshire, J. 1978. Structural proteins of porcine enteroviruses. Vet. Microbiol. 3: 23-30.

Tattersall, P. 1972. Replication of the parvovirus mvm. I.

Dependence of virus multiplication and plaque formation on cell growth. J. Virol. 10: 586-590.

Tattersal, P. 1978a. Parvovirus protein structure and virion maturation. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 53-72, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Tattersall, P. 1978b. Susceptibility to minute virus of mice as a function of host-cell differentiation. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp.131-150, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Tattersall, P., Cawte, P. Shatkin, A. and Ward, D. 1976. Three structural polypeptides coded for by minute virus of mice, a parvovirus. J. Virol. 20: 273-289.

Tattersall, P. and Ward, D. 1976. Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. Nature 263: 106-109.

Tattersall, P. and Ward, D. 1978. The parvoviruses - an introduction. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 3-12, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Thiel, J. and Smith, K. 1967. Fluorescent focus assay on cell monolayers in plastic petri plates. Proc. Soc. Exp. Biol. Med. 125: 892-895.

Toolan, H. 1964. Studies on the h-viruses. Proc. Am. Assoc. Can. Res. 5: 64-75.

Toolan, H. 1978. Maternal role in susceptibility of embryonic and newborne hamsters to h-1 parvovirus. *In:* Replication of Mammalian Parvoviruses, edited by Ward, D. and Tattersall, P. pp. 161-176, Cold Spring Harbour Press, Cold Spring Harbour, N.Y.

Toolan, H., Dalldorf, G., Barclay, M., Chandra, S., and Moore, E. 1960. An unidentified, filtrable agent isolated from transplanted human tumors. Proc. Nat. Acad. Sci. 46: 1256-1263.

Usategui-Gomez, M. 1965. A human placental fluid inhibitor to haemagglutination by h-1 and h-b viruses: I. Purification. Proc. Soc. Exp. Biol. Med. 120: 385-390.

Verge, J. and Cristoforoni, N. 1928. La gastro-enterite infectieuse des chats. Est elle due a un des virus filtrable? C. R. Soc. Biol. Paris 99: 312-314.

Wadell, G. and de Jong, J. 1980. Restriction endonucleases in identification of a genome type of adenovirus 19 associated with keratoconjunctivitis. Infect. Immun. 27: 292-296.

Wills, C. 1952. Notes on infectious enteritis of mink and its relationship to feline enteritis. Can. J. Comp. Med. 16: 419-421.

Winberg, G. and Wadell, G. 1976. Structural polypeptides of adenovirus type 16 incomplete particles. J. Virol. 22: 389-401.